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(57) Abstract

The present invention provides isolated polypeptides, prolyl tripeptidyl–peptidases, and active analogs, active fragments or active modifications thereof, having amidolytic activity for cleavage of a peptide bond present in a target peptide having at least 30 amino acids. Isolated nucleic acid fragments encoding isolated prolyl tripeptidyl–peptidases are also provided, as are methods of reducing growth of a bacterium by inhibiting a prolyl tripeptidyl–peptidase.

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PROLYL PEPTIDASES AND METHODS OF USE

CONTINUING APPLICATION DATA

This patent application claims the benefit of U.S. provisional patent application No. 60/123,148, filed March 5, 1999, which is incorporated by reference herein.

GOVERNMENT FUNDING

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BACKGROUND OF THE INVENTION

Porphyromonas gingivalis (formerly *Bacteroides gingivalis*) is an obligately anaerobic bacterium which is implicated in periodontal disease. *P. gingivalis* produces several distinct proteolytic enzymes, many of which are recognized as important virulence factors. A number of physiologically significant proteins, including collagen, fibronectin, immunoglobulins, complement factors C3, C4, C5, and B, lysozyme, iron-binding proteins, plasma proteinase inhibitors, fibrin and fibrinogen, and factors of the plasma coagulation cascade system, are hydrolyzed by *P. gingivalis* proteases. Broad proteolytic activity plays a role in the evasion of host defense mechanisms and the destruction of gingival connective tissue in progressive periodontitis.

Progressive periodontitis is characterized by acute tissue degradation promoted by collagen digestion and a vigorous inflammatory response characterized by excessive neutrophil infiltration. Gingival crevicular fluid accumulates in periodontitis as periodontal tissue erosion progresses at the foci of the infection, and numerous plasma proteins are exposed to proteinases expressed by the bacteria at the injury site. Neutrophils are recruited to the gingiva, in part, by the humoral chemotactic factor C5a. The complement components C3 and C5 are activated by complex plasma proteases with "trypsin-like" specificities called convertases. The human plasma convertases

cleave the α-chains of C3 and C5 at a specific site generating biologically active factors known as anaphylatoxins (i.e. C3a and C5a). The anaphylatoxins are potent proinflammatory factors exhibiting chemotactic and/or spasmogenic activities as well as promoting increased vascular permeability. The larger products from C3 and C5 cleavage (i.e. C3b and C5b) participate in functions including complement cascade activation, opsonization, and lytic complex formation.

Recent studies have indicated that this periodontopathogen produces at least seven different enzymes belonging to the cysteine and serine catalytic classes of peptidases, among which three cysteine proteinases (gingipains) are predominant (Potempa, J., et al. (1995) *Prospect. Drug Discovery and Design* 2, 445-458). The gingipains are the best characterized group of *P. gingivalis* enzymes as their structure, function, enzymatic properties and pathological significance are known. From *in vitro* studies it is apparent that two gingipains R (also referred to generally as "Arg-gingipains" and more specifically as RgpA and RgpB), enzymes specific for cleavage at Arg-Xaa peptide bonds, have a significant potential to contribute to the development and/or maintenance of a pathological inflammatory state in infected periodontal pockets through: (*i*) activation of the kallikrein-kinin cascade, (*ii*) the release of neutrophil chemotactic activity from native and oxidized C5 of the complement pathway, and (*iii*) activation of factor X. In addition, gingipain K (also referred to as "Lys-gingipain"), an enzyme which cleaves Lys-Xaa peptide bonds, degrades fibrinogen. This may add to a bleeding on probing tendency associated with periodontitis. Finally, the presence of a hemagglutinin/adhesion domain in the non covalent multiprotein complexes of RgpA and gingipain K suggests participation of these enzymes in the binding of *P. gingivalis* to extracellular matrix proteins which may facilitate tissue invasion by this pathogen.

In comparison to the gingipains, relatively little is known about other cysteine proteinases produced by *P. gingivalis*. Two genes, referred to as *tpr* and *prtT* have been cloned and sequenced and although they encode a putative papain-like and streptopain-like cysteine proteinases, respectively, neither has been purified and characterized.

The presence of serine proteinase activity in cultures of *P. gingivalis* has been known for several years; however, only limited information is available about such enzymes. Indeed, a serine endopeptidase has been isolated from culture media, although it was only superficially characterized (Hinode D., et al., (1993) *Infect. Immun.* 59, 3060-3068). On the other hand, an enzyme referred to as glycylprolyl peptidase (DPP IV) was found to be associated with bacterial surfaces and two molecular mass forms of this peptidase have been described. This enzyme has also been shown to possess the ability to hydrolyze partially degraded type I collagen, releasing the Gly-Pro dipeptide, and it was suggested that, in collaboration with collagenase, glycylprolyl peptidase may contribute to the destruction of the periodontal ligament (Abiko, Y., et al. (1985) *J.Dent. Res.* 64, 106-111). In addition to this potential pathological function, glycylprolyl peptidase may also play a vital role in providing *P. gingivalis* with dipeptides which can be transported inside the cell and serve as a source of carbon, nitrogen, and energy for this asaccharolytic organism. Recently, a gene encoding glycylprolyl peptidase in *P. gingivalis* has been cloned and sequenced, and it is now apparent that this enzyme is homologous to dipeptidyl-peptidase IV (DPP-IV) from other organisms (Kiyama, M., et al. (1998) 1396, 39-46). The nucleotide sequence of the genome of this bacterium is currently being determined by The Institute for Genomic Research, and is available at www.tigr.org.

SUMMARY OF THE INVENTION

The present invention is directed to an isolated prolyl tripeptidyl-peptidase, active analog, active fragment, or active modification thereof having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids. Alternatively, the isolated prolyl tripeptidyl-peptidase, active analog, active fragment, or active modification thereof is isolated from *P. gingivalis*. Typically, amidolytic activity is determined with a prolyl tripeptidyl-peptidase:target polypeptide ratio of at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours. The peptide cleaved by the isolated prolyl tripeptidyl-peptidase can include the sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID

NO:7, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:11, H-Ala-Arg-Pro-Ala-D-Lys-amide, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:25, or SEQ ID NO:37. The amino acid sequence of the isolated prolyl tripeptidyl-peptidase can include the amino acid sequence GXSXXG (SEQ ID NO:39), the amino acid sequence GXSXGG (SEQ ID NO:40), or the amino acid sequence of SEQ ID NO:30.

Another aspect of the invention is an isolated polypeptide, active analog, active fragment, or active modification thereof having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids. Typically, the polypeptide:target polypeptide ratio of at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours.

The invention is also directed to an isolated polypeptide comprising an amino acid sequence having a percentage amino acid identity of greater than 35 % with SEQ ID NO:30.

An alternative aspect of the invention is an isolated nucleic acid fragment encoding a prolyl-tripeptidyl peptidase, active analog, active fragment, or active modification thereof, having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids. Typically, the prolyl tripeptidyl-peptidase:target polypeptide ratio of at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours. The nucleic acid fragment can have a nucleotide sequence comprising SEQ ID NO:38. A complement of the nucleic acid fragment can hybridize to SEQ ID NO:38 under hybridization conditions of 0.5 M phosphate buffer, pH 7.2, 7 % SDS, 10 mM EDTA, at 68°C, followed by three for 20 minutes washes in 2x SSC, and 0.1 % SDS, at 65°C, wherein at least about 20 nucleotides of the complement hybridize.

Another aspect of the invention is an isolated nucleic acid fragment encoding a polypeptide that includes an amino acid sequence having a percentage amino acid identity of greater than 35 % with SEQ ID NO:30.

The invention is also directed at a method of identifying an inhibitor of a prolyl-tripeptidyl peptidase, active analog, active fragment, or active modification thereof, including identifying a molecule that inhibits the

amidolytic activity of the prolyl-tripeptidyl peptidase. The inhibitor is identified by incubating the prolyl-tripeptidyl peptidase with the molecule under conditions that promote amidolytic activity of the prolyl-tripeptidyl peptidase and determining if the amidolytic activity of the prolyl-tripeptidyl peptidase is inhibited relative to the amidolytic activity in the absence of molecule.

An aspect of the invention is a method of reducing growth of a bacterium. This method includes inhibiting a prolyl tripeptidyl-peptidase, active analog, active fragment, or active modification thereof, or a prolyl dipeptidyl-peptidase, active analog, active fragment, or active modification thereof. The method includes contacting the prolyl tripeptidyl-peptidase with an inhibitor of the prolyl tripeptidyl-peptidase. The method can be used to protect an animal from a periodontal disease caused by *P. gingivalis* including administering to the animal the inhibitor. The disease can be selected from the group consisting of gingivitis and periodontitis. The inhibitor can be administered by a method selected from the group consisting of subgingival application and controlled release delivery.

Another aspect of the invention is an immunogenic composition including an isolated prolyl tripeptidyl-peptidase, or an antigenic analog, antigenic fragment, or antigenic modification thereof, the prolyl tripeptidyl-peptidase having amidolytic activity for cleavage of a peptide bond present in a target peptide having at least 4 amino acids. Typically, the prolyl tripeptidyl-peptidase:target polypeptide ratio is at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours. The immunogenic composition can include an adjuvant.

The invention is also directed to a composition including an inhibitor of an isolated prolyl tripeptidyl-peptidase and a pharmaceutically acceptable carrier.

Additional aspects of the invention include a dipeptidyl peptidase having an amino acid sequence including SEQ ID NO:43, SEQ ID NO:44, or SEQ ID NO:45.

Definitions

"Polypeptide" as used herein refers to a polymer of amino acids and does not refer to a specific length of a polymer of amino acids. Thus, for example, the terms peptide, oligopeptide, protein, and enzyme are included within the definition of polypeptide. This term also includes post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations, and the like. A polypeptide can be produced by an organism, or produced using recombinant techniques, or chemically or enzymatically synthesized.

"Peptidase," "proteinase," and "protease" all refer to enzymes that catalyze the hydrolysis of peptide bonds in a polypeptide. A "peptide bond" or "amide bond" is a covalent bond between the alpha-amino group of one amino acid and the alpha-carboxyl group of another amino acid. "Peptidase inhibitor," "proteinase inhibitor," "protease inhibitor," and "inhibitor" all refer to molecules that inhibit a peptidase that catalyzes the hydrolysis of peptide bonds in a polypeptide.

As used herein, the term "isolated" means that a polypeptide or a nucleic acid fragment has been either removed from its natural environment, produced using recombinant techniques, or chemically or enzymatically synthesized. Preferably, the polypeptide or nucleic acid fragment is purified, i.e., essentially free from any other polypeptides or nucleic acid fragments and associated cellular products or other impurities.

"Amidolytic activity" refers to the ability of a polypeptide to catalyze the hydrolysis of at least one peptide bond in a polypeptide. The term "cleavage" can also be used to refer to the hydrolysis of a peptide bond in a polypeptide. "Prolyl-tripeptidyl peptidase" and "PTP" refer to a polypeptide having a particular "amidolytic activity". A "prolyl-tripeptidyl peptidase" is able to hydrolyze the peptide bond between the proline and the Yaa residues in a target polypeptide with the general formula NH_2-Xaa-Xaa-Pro-Yaa-$(Xaa)_n$ (SEQ ID NO:25), wherein Xaa is a natural or modified amino acid, Yaa is a natural or modified amino acid except proline, and the α-amino of the amino terminal residue is not blocked. A "prolyl tripeptidyl-peptidase" does not have to cleave all members of the target peptide. The term "natural amino acid" refers to the 20 amino acids typically produced by a cell. The term "modified amino acid"

refers to, for instance, acetylation, hydroxylation, methylation, amidation, and the attachment of carbohydrate or lipid moieties, cofactors, and the like.

A "target polypeptide" is a polypeptide that is the potential substrate of the amidolytic activity of a prolyl tripeptidyl-peptidase.

An active analog, active fragment, or active modification of a polypeptide of the invention is one that has amidolytic activity by hydrolysis of a peptide bond present in the target polypeptide as described herein. Active analogs, fragments, and modifications are described in greater detail herein.

"Nucleic acid fragment" as used herein refers to a linear polymeric form of nucleotides of any length, either ribonucleotides or deoxynucleotides, and includes both double- and single-stranded DNA and RNA. A nucleic acid fragment may include both coding and non-coding regions that can be obtained directly from a natural source (e.g., a microorganism), or can be prepared with the aid of recombinant or synthetic techniques. A nucleic acid molecule may be equivalent to this nucleic acid fragment or it can include this fragment in addition to one or more other nucleotides or polynucleotides, For example, the nucleic acid molecule of the invention can be a vector, such as an expression of cloning vector.

"Percentage amino acid identity" refers to a comparison of the amino acids of two polypeptides as described herein.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1. Purification of the prolyl tripeptidyl peptidase from the acetone precipitate of the *P. gingivalis* cell extracts. Absorbance at 280 nm (open triangles), amidolytic activity against H-Ala-Phe-Pro-pNA (closed diamonds), and H-Gly-Pro-pNA (closed circles). *(a)* Separation of PTP-A on hydroxyapatite. *(b)* Separation of PTP-A on Phenyl-Sepharose HP. *(c)* Separation of PTP-A on MonoQ FPLC. *(d)* Chromatofocusing of PTP-A on Mono-P.

Fig. 2. SDS -PAGE of fractions from purification of PTP-A and the autoradiography of the purified enzyme. *Lane a*, molecular mass markers (phosphorylase B, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa; α-lactalbumin, 14

kDa); *lane b*, acetone precipitate from Triton X-100 extract of *P. gingivalis*, *lane c*: hydroxyapatite column eluate; *lane d*, Phenyl-Sepharose column eluate; *lane e*, MonoQ column eluate; *lane f*, purified PTP-A from MonoP column wash; *lane g*, autoradiograph of ^{3}H-DFP labeled enzyme exposed for 96 h to X-ray film. All samples were reduced and boiled prior to PAGE analysis.

Fig. 3. Multiple sequence alignment of *P. gingivalis* PTP-A (PTP-A) and its bacterial and eukaryotic homologues. Pg-DPP, DPP from *P. gingivalis* (Kiyama, M., et al., (1998) *Bioch. Bioph. Acta* 1396, 39-46) containing an amino-terminal sequence corrected according to the *P. gingivalis* W83 genome data available from The Institute of Genomic Research at www.tigr.org); Fm-DPP, DPP from *Flavobacterium meningosepticum*; Hs-DPP, human DPP IV; and Mm-FAP, mouse fibroblast activation protein. Peptide sequences obtained from PTP-A analysis described herein are indicated with arrows (note that the sequence of the peptide 81-97 corresponds to the N-terminus of the lower molecular weight form of PTP-A); catalytic triad is marked with asterisks; and the proposed PTP-A membrane-anchoring N–terminal α-helix is double-underlined. Homologous regions (i.e., regions of identical amino acids and/or conservative substitutions) are highlighted. Identical regions are shown as white letters on a black background.

Fig. 4. Comparison of *P. gingivalis* PTP-A and DPP active site domains to corresponding sequences of three putative homologues identified within the *P. gingivalis* genome (DPP-H1, DPP-H2 and DPP-H3). Sequences of *P. gingivalis* PTP-A, DPP, DPP-H1, DPP-H2, and DPP-H3 were obtained from conceptual translation of the following open reading frames retrieved from The Institute for Genomic Research (TIGR) unfinished P. gingivalis genome database: gnl | TIGR | *P. gingivalis* contig 126 (positions 13 228 – 15 426), contig 87 (positions 6 424 – 4 399), contig 65 (positions 161 – 1 786), contig 101 (positions 8 895 – 6 845), and contig 9 (positions 4 216 – 2 162), respectively. Residues predicted as catalytic triads are marked with asterisks. Homologous regions (i.e., regions of identical amino acids and/or conservative substitutions) are highlighted.

Identical regions are shown as white letters on a black background. Similar regions (i.e., conservative substitutions) are shown as white letters on a grey background.

Fig. 5. Influence of Pefabloc-serine proteinase inhibitor on *P. gingivalis* growth.

Fig. 6. Comparison of *P. gingivalis* PTP-A and DPP to sequences of three putative homologues identified within the *P. gingivalis* genome (DPP-H1, DPP-H2 and DPP-H3). Sequences of *P. gingivalis* PTP-A (126PP), DPP (87PP), DPP-H1 (65PP), DPP-H2 (101PP), and DPP-H3 (9PP) were obtained as described in Fig. 4. Homologous regions (i.e., regions of identical amino acids and/or conservative substitutions) are highlighted. Identical regions are shown as white letters on a black background. Similar regions (i.e., conservative substitutions) are shown as white letters on a grey background.

Fig. 7. Nucleotide sequence (SEQ ID NO:38) and amino acid sequence (SEQ ID NO:30) of PTP-A.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides isolated polypeptides, preferably isolated prolyl peptidases, more preferably prolyl dipeptidyl-peptidases and prolyl tripeptidyl-peptidases, most preferably prolyl-tripeptidyl peptidases, that have amidolytic activity by hydrolysis of a peptide bond present in a target polypeptide, where the bond is between a proline and an amino acid residue attached to the alpha-carboxyl group end of the proline.

When the prolyl peptidase is a prolyl tripeptidyl-peptidase, the peptidase has amidolytic activity by hydrolysis of a peptide bond present in a target polypeptide of the general formula NH_2-Xaa-Xaa-Pro-Yaa-$(Xaa)_n$ (SEQ ID NO:25), wherein Xaa is a natural or modified amino acid, Yaa is a natural or modified amino acid except proline, and the α-amino of the amino terminal residue is not blocked, wherein the peptide bond of the target polypeptide that is hydrolyzed is the Pro-Yaa peptide bond. Preferably, isolated polypeptides do not cleave a target peptide having a blocked α-amino of the amino terminal residue.

Preferably, the only peptide bond of the target peptide that is hydrolyzed is the Pro-Yaa bond. In increasing order of preference, isolated polypeptides can cleave a target peptide that is at least 4 amino acids or at least 300 Da, at least 10 amino acids or at least 750 Da, at least 20 amino acids or at least 1,500 Da, or at least 30 amino acids or at least 3,000 Da. Preferably, the prolyl-tripeptidyl peptidases cleave peptides 1, 2, 7, 8, and 10-15 shown in Table 3, human cystatin C, and interleukin 6.

When the prolyl peptidase is a prolyl dipeptidyl-peptidase, the peptidase has amidolytic activity by hydrolysis of a peptide bond present in a target polypeptide of the general formula NH_2-Xaa-Zaa-Yaa-$(Xaa)_n$ (SEQ ID NO:12), wherein Xaa is a natural or modified amino acid, Zaa is a proline or alanine, Yaa is a natural or modified amino acid except proline or hydroxyproline, and the α-amino of the amino terminal residue is not blocked, wherein the peptide bond of the target polypeptide that is hydrolyzed is the Zaa-Yaa peptide bond. Preferably, isolated polypeptides does not cleave a target peptide having a blocked α-amino of the amino terminal residue. Preferably, the only peptide bond of the target peptide that is hydrolyzed is the Pro-Yaa bond.

Due to their cyclic aliphatic character proline residues bestow unique conformational constraints on polypeptide chain structures, significantly affecting the susceptibility of proximal peptide bonds to proteolytic cleavage. Those proline residues, which often appear near the amino-termini of many biologically active peptides, may protect them against proteolytic degradation by peptidases with general specificity. A specialized group of proteolytic enzymes, typically referred to as prolyl peptidases, has evolved to cleave (i.e., hydrolyze) a peptide bond adjacent to a proline residue in a polypeptide. The peptide bond adjacent to a proline residue can be referred to as a prolyl-X bond, where prolyl is the proline residue, and X is an amino acid residue attached to the alpha-carboxyl group end of the proline. The bacterial prolyl peptidases can cleave a polypeptide to liberate a tripeptide or a dipeptide. Prolyl peptidases that do not cleave a target peptide if the α-amino of the amino terminal residue is blocked can be referred to as exopeptidases. The *in vivo* activity of these specialized proteolytic enzymes may have important physiological significance, because it may lead to inactivation of many biologically active peptides and/or transformation of the activity of other

biologically active peptides. In addition, hydrolysis of prolyl-X bonds in conjunction with general catabolic pathways should allow the complete re-utilization of amino acids by living organisms, including bacteria. However, prolyl peptidases from bacterial pathogens, if released into the host environment, may interfere with the physiological functions of biologically active polypeptides and, therefore, contribute to the pathogenicity of infectious disease.

The external (i.e., cell surface) localization and uncontrolled activity of bacterial peptidases, including prolyl peptidases, likely contributes significantly to run-away inflammation in the human host and the pathological degradation of connective tissue during periodontitis. For instance, working in concert bacterial prolyl peptidases (e.g., prolyl tripeptidyl peptidases and DPP IV) have the ability to completely degrade collagen fragments locally generated by endogenous or bacterial collagenases. Because type I collagen is the major component of periodontal ligament, its enhanced degradation by bacterial prolyl peptidases may contribute to loss of tooth attachment and periodontal pocket formation. Thus, there is a need in the art to characterize bacterial peptidases to facilitate the development of therapies to inhibit the activity of the bacterial peptidases.

The polypeptides of the present invention, preferably prolyl peptidases, can be used as a source of antibodies for inhibiting the peptidase activity and thereby possibly reducing periodontitis, loss of tooth attachment and periodontal pocket formation. Antibodies to prolyl peptidases can also be used to identify and/or isolate additional prolyl peptidases. Knowledge of prolyl peptidases can also be used to make inhibitors of prolyl peptidases and to make immunogenic compositions that could be used to elicit the production of antibodies to prolyl peptidases and thereby possibly reduce gingivitis, periodontitis, loss of tooth attachment, and/or periodontal pocket formation.

An example of a prolyl-tripeptidyl peptidase is prolyl-tripeptidyl peptidase A (SEQ ID NO:30) (also referred to as PTP-A) from *P. gingivalis*. Purified PTP-A has apparent molecular masses of 81.8 and 75.8 kDa. The lower molecular mass peptidase may be due to the proteolytic cleavage of the peptidase from the surface of *P. gingivalis*. PTP-A is a new member of clan SC, family S9 of serine peptidases. Clans of serine peptidases are grouped on the basis of the order of certain amino acids in the polypeptide that make up the "catalytic triad" which

plays a pivotal role peptidase activity. The members of the clan SC are characterized by the catalytic triad in the polypeptide in the order of serine, aspartic acid, and histidine. Members of the clan SC are also characterized by a tertiary structure including β/α/β units, and an α/β hydrolase fold. In addition to the catalytic triad order, the amino acid sequence GXSXXG (SEQ ID NO:39), where X is any amino acid and S is the active site serine, is a signature of all members of the clan SC with some distinguishing features specific for each family. Family S9 has the consensus sequence GXSXGG (SEQ ID NO:40). Besides this consensus sequence, there is a general similarity of primary structures which classifies peptidases to this family. For instance, peptidases of this family generally have two domains, an amino-terminal domain that contains a membrane binding domain, and a carboxy-terminal domain, also referred to as the catalytic domain. The catalytic domain contains the residues of the catalytic triad. Some members of the S9 family have only the catalytic domain.

The S9 family is diverged and divided in three subfamilies: S9A, cytosolic oligopeptidases from archae and eukaryotes; S9B, eukaryotic acylaminoacylpeptidases; and S9C, dipeptidyl peptidease IV from bacteria and eukaryotes. The catalytic domain of peptidases from family S9 typically begin at about residue 400 of SEQ ID NO:30 and include the remaining carboxy-terminal amino acids (see, e.g., Fulop, et al., (1998) *Cell* 94, 161-170). Despite structural similarities to peptidases from the S9 family, the tripeptidyl-peptidase activity of PTP-A is unusual for this family of enzymes, and no other known similar activity has so far been attributed to any other member of the S9 family. In fact, all strict tripeptidyl-peptidases belong only to the subtilisin family (S8) and S33 family of serine peptidases; however, they neither share a structural relationship with PTP-A nor have activity limited to cleavage after proline residues. In particular, there are no other known prolyl tripeptidyl peptidases with an activity that is increased by iodoacetamide relative to the same prolyl tripeptidyl peptidase in the absence of iodoacetamide under the same conditions. Iodoacetamide is a compound that is traditionally a peptidase inhibitor. Typically, the activity of a prolyl tripeptidyl peptidase is increased about two-fold. Furthermore, unlike oligopepidases, the prolyl tripeptidyl-peptidases of the present invention can cleave target peptidases having as few as 4 amino acids but also target peptides having at least 30 amino

acids or a molecular weight of at least 3,000 Da. In these respects, the *P. gingivalis* tripeptidyl peptidase is a unique enzyme, and the isolation and characterization of this novel bacterial prolyl peptidase will facilitate the development of therapies to inhibit the activity of the bacterial peptidases.

Examples of putative prolyl-dipeptidyl peptidases are DPP-H1 (SEQ ID NO:43), DPP-H2 (SEQ ID NO:44), and DPP-H3 (SEQ ID NO:45). These peptidases have a significant percentage amino acid similarity with DPP IV and PTP-A (see Fig. 6). Each dipeptidyl peptidase is expected to have enzymatic activity, as each has a well preserved catalytic triad (Fig. 4). DPP IV has been characterized and the gene encoding the peptidase has been cloned, however the substrate specificity has not been well characterized. DPP IV has been found to cleave SEQ ID NOs:6, 20, 23, and 24. DPP IV has been purified in two forms. One of the forms is a full length gene translation product containing a blocked amino-terminal residue. The second form had the amino-terminal amino acid sequence HSYRAAVYDYDVRRNLVKPLSEHVG (SEQ ID NO:48), which corresponds to residues 116-140 of DPP IV (Kiyama, M., et al. (1998) 1396, 39-46), indicating that it was proteolytically truncated on the amino-terminus.

In *P. gingivalis*, PTP-A and DPP IV activity is cell surface associated. While not intending to be limiting, it is conceivable that the enzyme is membrane anchored through a putative signal sequence which is not cleaved but remains as a membrane spanning domain similar to other members of the prolyl oligopeptidase family. However, a significant portion of the purified PTP-A has a truncated N-terminus, apparently due to cleavage by Lys-specific peptidase and likely to be an artifact which has occurred during the purification procedure. Nevertheless, membrane bound PTP-A and DPP IV is proteolytically cleaved and shed during cultivation of the bacteria, as indicated by variable amount of soluble activities found in cell free culture media. The cell surface localization of PTP-A supports a putative physiological function in providing nutrients for growing bacterial cells. The inability of asaccharolytic *P. gingivalis* to utilize free amino acids makes the bacterium entirely dependant on an external peptide supply. In this regard, PTP-A and DPP IV activities are probably very important, if not indispensable, for bacterial growth, and inhibition of prolyl tripeptidyl-peptidases and dipeptidyl-peptidases may inhibit the *in vivo* growth of organisms, including *P. gingivalis*.

For instance, treatment of *P. gingivalis* cultures in lagphase (i.e., the period after inoculation of a culture and before the organism begins to divide) and early logarithmic growth with the inhibitors PEFABLOCK and 3,4-dichloroisocoumarin inhibits growth of *P. gingivalis*.

Preferably, a polypeptide of the invention, preferably a prolyl peptidase, contains the amino acid sequence GXSXXG (SEQ ID NO:39), most preferably, GXSXGG (SEQ ID NO:40), where G is glycine, X is any amino acid, and S is the active site serine. The active site serine can be identified by, for instance, labeling with diisopropylfluorophosphate as described herein. Preferably, the catalytic domain of the prolyl tripeptidyl-peptidases of the invention begins at about residue 400 of SEQ ID NO:30 and includes the remaining carboxy-terminal amino acids and the corresponding amino acids of SEQ ID NOs:43-45 (see Fig. 6), more preferably, at about residue 502 of SEQ ID NO:30 and includes the remaining carboxy-terminal amino acids and the corresponding amino acids of SEQ ID NOs:43-45 (see Fig. 6), most preferably, at about residue 556 of SEQ ID NO:30 and includes the remaining carboxy-terminal amino acids and the corresponding amino acids of SEQ ID NOs:43-45 (see Fig. 6).

The invention further includes a polypeptide, preferably a prolyl tripeptidyl-peptidase, that shares a significant level of primary structure with SEQ ID NO:30. The two amino acid sequences (i.e., the amino acid sequence of the polypeptide and the sequence SEQ ID NO:30) are aligned such that the residues that make up the catalytic triad, i.e., the serine, aspartic acid, and the histidine, are in register, then further aligned to maximize the number of amino acids that they have in common along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to place the residues of the catalytic triad in register and to maximize the number of shared amino acids, although the amino acids in each sequence must nonetheless remain in their proper order. The percentage amino acid identity is the higher of the following two numbers: (a) the number of amino acids that the two sequences have in common within the alignment, divided by the number of amino acids in SEQ ID NO:30, multiplied by 100; or (b) the number of amino acids that the two sequences have in common within the alignment, divided by the number of amino acids in the candidate polypeptide, multiplied by 100. Preferably, a prolyl tripeptidyl peptidase has

greater than 35 % identity, more preferably at least about 40 % identity, most preferably at least about 45 % identity with SEQ ID NO:30. Preferably, amino acids 154-732 of SEQ ID NO:30 are used, more preferably amino acids 400-732 of SEQ ID NO:30 are used. An isolated polypeptide comprising an amino acid sequence having a percentage amino acid identity of greater than 35 % with SEQ ID NO:30.

In general, the amidolytic activity of the polypeptides of the invention, preferably prolyl peptidases, can be measured by assay of the cleavage of a target polypeptide in the presence of prolyl peptidase and a buffer. Preferably, the lower ratio of prolyl tripeptidyl-peptidase to target polypeptide is at least about 1:1, more preferably at least about 1:100, even more preferably at least about 1:1,000, most preferably at least about 1:10,000. Preferably, the higher ratio of prolyl peptidase to target polypeptide is no greater than about 1:10,000,000, more preferably no greater than about 1:1,000,000 and most preferably no greater than about 1:100,000. Buffers in which a prolyl peptidase is active are suitable for the assay. Preferably, the buffer is about 200 mM HEPES (N-2-hydroxyethylpeperazine,N'-2-ethansulfonic acid), more preferably about 50 mM HEPES, most preferably about 20 mM HEPES. Preferably, the pH of the buffer is at least about pH 6.0 and no greater than pH 8.0, more preferably about pH 7.5. Preferably, the temperature of the assay is at about 37°C. The assay can be carried out for at least about 1 minute to no greater than 24 hours. Preferably, the amidolytic activity of the prolyl peptidases are measured at a prolyl peptidase:target polypeptide ratio of at least about 1:100 to no greater than 1:1,000,000 in about 200 mM HEPES, about pH 7.5 at about 37°C for at least about 3 hours. In general, the time of the assay can vary depending on the substrate and enzyme:substrate ratio. Typically, target peptides are stable under these conditions, and typically it is difficult to detect background levels of hydrolysis in the absence of a prolyl peptidase. Preferably, the assay is allowed to continue until at least 1 % of the target peptide is hydrolyzed.

Prolyl-tripeptidyl peptidases of the present invention preferably are inhibited by a compound chosen from the group consisting of PEFABLOCK (4-(2-aminoethyl)-benzenesulfonyl-fluoride hydrochloride), diisopropylfluorophosphate, and 3,4-dichloroisocoumarin, more preferably PEFABLOCK and diisopropylfluorophosphate, and most preferably diisopropylfluorophosphate. The

peptidases of the present invention are preferably not inhibited by a compound chosen from the group consisting of leupeptin, antipain, E-64, pepstatin, α_1-proteinase inhibitor, α_1–antichymotrypsin and α_2–macroglobulin, most preferably. Significantly and unexpectedly, the amidolytic activity of a prolyl-tripeptidyl peptidase of the present invention is increased by iodoacetamide relative to the prolyl-tripeptidyl peptidase in the absence of iodoacetamide under the same conditions. Preferably, the effect of iodoacetamide on amidolytic activity is measured by incubating in 200 mM HEPES, pH 7.6, at least about 0.1 nM of the prolyl tripeptidyl-peptidase with the inhibitor for about 15 minutes, adding about 1 mM of H-Ala-Phe-Pro-pHA, and incubating for at least about 1 minute before assaying for amidolytic activity. Typically, at least about 1 mM to no greater than 100 mM of inhibitor is used.

The polypeptides of the invention include a polypeptide having SEQ ID NO:30, or an active analog, active fragment, or active modification of SEQ ID NO:30. An active analog, active fragment, or active modification of a polypeptide having SEQ ID NO:30 is one that has amidolytic activity by hydrolysis of the Pro-Yaa peptide bond present in a target polypeptide of the general formula NH_2-Xaa-Xaa-Pro-Yaa-$(Xaa)_n$ (SEQ ID NO:25). Active analogs of a polypeptide having SEQ ID NO:30 include prolyl-tripeptidyl peptidases having amino acid substitutions that do not eliminate hydrolysis of SEQ ID NO:25 at the Pro-Yaa peptide bond. Substitutes for an amino acid may be selected from other members of the class to which the amino acid belongs. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and tyrosine. Polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Examples of preferred conservative substitutions include Lys for Arg and *vice versa* to maintain a positive charge; Glu for Asp and *vice versa* to maintain a negative charge; Ser for Thr so that a free -OH is maintained; and Gln for Asn to maintain a free NH_2.

Active fragments of a prolyl-tripeptidyl peptidase of the invention include prolyl-tripeptidyl peptidases containing deletions or additions of one or more contiguous or noncontiguous amino acids such that the resulting polypeptide will

hydrolyze SEQ ID NO:25 at the Pro-Yaa peptide bond. An example of a fragment of a prolyl-tripeptidyl peptidase is a catalytic domain. Modified prolyl-tripeptidyl peptidases include prolyl-tripeptidyl peptidases that are chemically and enzymatically derivatized at one or more constituent amino acid, including side chain modifications, backbone modifications, and N- and C- terminal modifications including acetylation, hydroxylation, methylation, amidation, and the attachment of carbohydrate or lipid moieties, cofactors, and the like. Modified prolyl-tripeptidyl peptidases will hydrolyze SEQ ID NO:25 at the Pro-Yaa peptide bond.

Prolyl peptidases can be obtained by several methods. Isolation of a prolyl-tripeptidyl peptidase present on the surface of a cell producing the peptidase typically requires lysis of the cell followed by purification methods that are well known in the art. Alternatively, cels can be treated with a detergent, for instance Triton X-100, to remove the peptidase from the cell surface. The following are nonlimiting examples of suitable protein purification procedures: fractionation on immunoaffinity, ion-exchange, hydroxyapatite, Phenyl-Sepharose HP, MonoQ HR 5/5, or MonoP columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on an ion-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75. Preferably, isolation of a prolyl-tripeptidyl peptidase from *P. gingivalis* is accomplished using a combination of hydroxyapatite, Phenyl-Sepharose HP, MonoQ HR 5/5 and MonoP column chromatography steps as described herein.

Prolyl peptidases can also be isolated from organisms other than *P. gingivalis*. Other organisms can express a prolyl-tripeptidyl peptidase that is encoded by a coding region having similarity to the PTP-A coding region. A "coding region" is a linear form of nucleotides that encodes a polypeptide, usually via mRNA, when placed under the control of appropriate regulatory sequences. The boundaries of a coding region are generally determined by a translation start codon at its 5' end and a translation stop codon at its 3' end. "Regulatory region" refers to a nucleic acid fragment that regulates expression of a coding region to which a regulatory region is operably linked. Non limiting examples of regulatory regions include promoters, transcription initiation sites, translation start sites, translation

stop sites, and terminators. "Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A regulatory element is "operably linked" to a coding region when it is joined in such a way that expression of the coding region is achieved under conditions compatible with the regulatory region. Alternatively, other organisms can express a prolyl-tripeptidyl peptidase from a recombinant coding region encoding the peptidase. The identification of similar coding regions in other organisms can be accomplished as described herein. A prolyl-tripeptidyl peptidase can be isolated using purification methods that are well known in the art. Alternatively, the peptidase can be chemically synthesized using methods that are well known in the art including, for instance, solid phase synthesis. Examples of, for instance, coding and regulatory regions are described herein.

The expression of a prolyl-tripeptidyl peptidase by an organism other than *P. gingivalis* can be detected using specific substrates of the general formula NH_2-Xaa-Xaa-Pro-LG or NH_2-Xaa-Xaa-Pro-Yaa (SEQ ID NO:41), where LG is a leaving group. The leaving group can be a chromogenic or fluorogenic group known to the art. The expression of a prolyl-tripeptidyl peptidase by an organism and subsequent cleavage of a specific substrate results in a free amino acid or a free leaving group, each of which can be assayed using techniques known to those of skill in the art. Other methods can be based on immunogenic properties of PTP-A, for instance immunoassays and histochemistry, the detection of mRNA, and PCR related methods, all of which are known to one of skill in the art.

As described in the Examples, the amino acid sequence of the amino-terminal end of a PTP-A fragment was used to identify the nucleotide sequence of the PTP coding region. The nucleotide sequence was present in a publically available database containing the nucleotide sequence of the partially finished *P. gingivalis* W83 genome. However, even though the nucleotides that encode the *P. gingivalis* PTP-A were known, there was no indication that the nucleotides were in fact transcribed and translated. The data obtained from the database only contained the nucleotide sequence of a genomic clone; there was no disclosure that the nucleotides did or did not contain an open reading frame. Moreover, there is little data known to the art regarding regulatory regions required for either the transcription or the translation of a nucleotide sequence in *P. gingivalis*.

Thus, a person of ordinary skill, having the nucleotide sequence of the genomic clone, would not be able to predict that the open reading frame encoding PTP-A was transcribed or translated. Moreover, even if there was a suggestion that the open reading frame was both transcribed and translated, there is no suggestion that the polypeptide encoded by the open reading frame would have the novel activity of PTP-A.

Accordingly, the present invention is directed to a nucleic acid fragment encoding a polypeptide, particularly a prolyl-tripeptidyl peptidase, active analog, active fragment, or active modification thereof. The nucleic acid fragment can have a nucleotide sequence as shown in SEQ ID NO:38. Alternatively, nucleic acid fragments of the invention include those whose complement hybridize to SEQ ID NO:38 under standard hybridization conditions as described herein. During hybridization the entire nucleotide sequence of the complement can hybridize with SEQ ID NO:38. Preferably, at least about 20 nucleotides of the complement hybridize with SEQ ID NO:38, more preferably at least about 50 nucleotides, most preferably at least about 100 nucleotides.

Alternatively, the nucleic acid fragment can have a nucleotide sequence encoding a polypeptide having the amino acid sequence shown in SEQ ID NO:30. An example of the class of nucleotide sequences encoding such a polypeptide is SEQ ID NO:38. This class of nucleotide sequences is large but finite, and the nucleotide sequence of each member of the class can be readily determined by one skilled in the art by reference to the standard genetic code.

The identification of similar coding regions in other organisms can be accomplished by screening individual wild-type microorganisms for the presence of nucleotide sequences that are similar to the coding region of PTP-A, which is shown in SEQ ID NO:38. Screening methods include, for instance, hybridization of a detectably labeled probe with a nucleic acid fragment.

Standard hybridizing conditions are a modification of the conditions used by Church et al. ((1984) *Proc. Natl. Acad. Sci. USA* 81, 1991): 0.5 M phosphate buffer, pH 7.2, 7 % SDS, 10 mM EDTA, at 68°C, and three washes, each for 20 minutes in 2x SSC, 0.1 % SDS, at 65°C. Preferably, a probe will hybridize to the nucleotide sequence set forth in SEQ ID NO:38 under standard hybridizing conditions. Generally the probe does not have to be complementary to all the

nucleotides of the nucleic acid fragment as long as there is hybridization under the above-stated conditions.

"Complement" and "complementary" refer to the ability of two single stranded nucleic acid fragments to base pair with each other, where an adenine on one nucleic acid fragment will base pair to a thymine on a second nucleic acid fragment and a cytosine on one nucleic acid fragment will base pair to a guanine on a second nucleic acid fragment. Two nucleic acid fragments are complementary to each other when a nucleotide sequence in one nucleic acid fragment can base pair with a nucleotide sequence in a second nucleic acid fragment. For instance, 5'-ATGC and 5'-GCAT are complementary. The term complement and complementary also encompasses two nucleic acid fragments where one nucleic acid fragment contains at least one nucleotide that will not base pair to at least one nucleotide present on a second nucleic acid fragment. For instance the third nucleotide of each of the two nucleic acid fragments 5'-ATTGC and 5'-GCTAT will not base pair, but these two nucleic acid fragments are complementary as defined herein. Typically two nucleic acid fragments are complementary if they hybridize under the standard conditions referred to herein.

Preferred probes are nucleic acid fragments complementary to a coding region or another nucleotide sequence that encodes a prolyl-tripeptidyl peptidase. For instance, a probe can comprise a consecutive series of nucleotides complementary to a portion of SEQ ID NO:38. Preferably a probe is at least about 18 bases, more preferably at least about 21 bases, and most preferably at least about 24 bases in length. Particularly preferred probes are TTCGATCCGGCAAAGAAATATCCTGTTATTGTCTATGTTTACGGAGGAC CT (SEQ ID NO:36, GTGGATGCCGATAGAATAGGAGTACATGGCTGGAGCTATGGTGGCTTT (SEQ ID NO:37, and SEQ ID NO:38. Methods of detectably labeling a probe are well known to the art.

The nucleic acid fragment that is identified by the probe is further analyzed to determine if it encodes a polypeptide with amidolytic activity of the Pro-Yaa

peptide bond on a target polypeptide of the general formula NH_2-Xaa-Xaa-Pro-Yaa-$(Xaa)_n$ (SEQ ID NO:25). Another method for screening individual microorganisms for the presence of nucleotide sequences that are similar to the coding regions of the present invention is the polymerase chain reaction (PCR).

Individual wild-type microorganisms containing nucleic acid fragments encoding a prolyl-tripeptidyl peptidase can also be identified using antibody. Preferably the antibody is directed to PTP-A. The production of antibodies to a particular polypeptide is known to a person of skill in the art, and is further detailed herein.

The use of hybridization of a probe to a coding region present in individual wild-type microorganisms can be used as a method to identify a coding region identical or similar to a coding region present in SEQ ID NO:38. The coding region can then be isolated and ligated into a vector as described below. Two nucleic acid sequences are "similar" if the two nucleic acid sequences can be aligned so that the number of identical amino acids along the lengths of their sequences are optimized. Preferably, two nucleotide acid sequences have, in increasing order of preference, preferably at least about 90 %, at least about 92 %, at least about 94%, at least about 96%, most preferably at least about 98% identity.

As mentioned above, a nucleic acid fragment of the invention can be inserted in a vector. Construction of vectors containing a nucleic acid fragment of the invention employs standard ligation techniques known in the art. See, e.g., Sambrook et al, *Molecular Cloning: A Laboratory Manual.*, Cold Spring Harbor Laboratory Press (1989) or Ausubel, R.M., ed. *Current Protocols in Molecular Biology* (1994). A vector can provide for further cloning (amplification of the nucleic acid fragment), i.e., a cloning vector, or for expression of the polypeptide encoded by the coding region, i.e., an expression vector. The term vector includes, but is not limited to, plasmid vectors, viral vectors, cosmid vectors, or artificial chromosome vectors. Typically, a vector is capable of replication in a bacterial host, for instance *E. coli*. Preferably the vector is a plasmid.

Selection of a vector depends upon a variety of desired characteristics in the resulting construct, such as a selection marker, vector replication rate, and the like. Suitable plasmids for expression in *E. coli*, for example, include pUC(X), pKK223-3, pKK233-2, pTrc99A, and pET-(X) wherein (X) denotes a vector family in which

numerous constructs are available. pUC(X) vectors can be obtained from Pharmacia Biotech (Piscataway, NH) or Sigma Chemical Co. (St. Louis, MO). pKK223-3, pKK233-2 and pTrc99A can be obtained from Pharmacia Biotech. pET-(X) vectors can be obtained from Promega (Madison, WI) Stratagene (La Jolla, CA) and Novagen (Madison, WI). To facilitate replication inside a host cell, the vector preferably includes an origin of replication (known as an "ori") or replicon. For example, ColE1 and P15A replicons are commonly used in plasmids that are to be propagated in *E. coli*.

An expression vector optionally includes regulatory regions operably linked to the coding region. The invention is not limited by the use of any particular promoter, and a wide variety are known. Promoters act as regulatory signals that bind RNA polymerase in a cell to initiate transcription of a downstream (3' direction) coding region. The promoter used in the invention can be a constitutive or an inducible promoter. It can be, but need not be, heterologous with respect to the host cell. Preferred promoters for bacterial transformation include *lac*, *lac*UV5, *tac*, *trc*, T7, SP6 and *ara*.

An expression vector can optionally include a Shine Dalgarno site (e.g., a ribosome binding site), and a start site (e.g., the codon ATG) to initiate translation of the transcribed message to produce the enzyme. It can also include a termination sequence to end translation. A termination sequence is typically a codon for which there exists no corresponding aminoacetyl-tRNA, thus ending polypeptide synthesis. The nucleic acid fragment used to transform the host cell can optionally further include a transcription termination sequence. The rrnB terminators, which is a stretch of DNA that contains two terminators, T1 and T2, is an often used terminator that is incorporated into bacterial expression systems (J. Brosius et al., (1981) *J. Mol. Biol.* 148 107-127).

The nucleic acid fragment used to transform the host cell optionally includes one or more marker sequences, which typically encode a polypeptide that inactivates or otherwise detects or is detected by a compound in the growth medium. For example, the inclusion of a marker sequence can render the transformed cell resistant to an antibiotic, or it can confer compound-specific metabolism on the transformed cell. Examples of a marker sequence are sequences that confer resistance to kanamycin, ampicillin, chloramphenicol, and tetracycline.

Antibodies can be produced to a polypeptide having the sequence of SEQ ID NOs:30, 43, 44 or 45, or a polypeptide having a percentage amino acid identity as described herein. Alternatively, antibodies can be made to an antigenic analog, antigenic fragment, or antigenic modification of a polypeptide having the sequence of SEQ ID NOs:30, 43, 44 or 45. An antigenic analog, antigenic fragment, or antigenic modification of a polypeptide having SEQ ID NOs:30, 43, 44 or 45 is one that generates an immune response in an animal. Antigenic analogs of a polypeptide having SEQ ID NOs:30, 43, 44 or 45 include prolyl peptidases having amino acid substitutions that do not eliminate peptide antigenicity in an animal. Substitutes for an amino acid may be selected from other members of the class to which the amino acid belongs, as described herein. Fragments of a prolyl peptidase of the invention include prolyl peptidases containing deletions or additions of one or more contiguous or noncontiguous amino acids such that the resulting polypeptide will generate an immune response in an animal. An example of a fragments of a prolylpeptidase is a catalytic domain. Modified prolyl peptidases include prolyl peptidases that are chemically and enzymatically derivatized at one or more constituent amino acids, including side chain modifications, backbone modifications, and N- and C- terminal modifications including acetylation, hydroxylation, methylation, amidation, and the attachment of carbohydrate or lipid moieties, cofactors, and the like.

Accordingly, an aspect of the invention is an immunogenic composition comprising an isolated prolyl peptidase, or an antigenic analog, antigenic fragment, or antigenic modification thereof, preferably a prolyl tripeptidyl-peptidase. The prolyl tripeptidyl-peptidase preferably has amidolytic activity for cleavage of the Pro-Yaa peptide bond present in a target polypeptide with the general formula NH_2-Xaa-Xaa-Pro-Yaa-$(Xaa)_n$ (SEQ ID NO:25), wherein the amidolytic activity is measured at a prolyl tripeptidyl-peptidase:target polypeptide ratio of at least about 1:100 to no greater than about 1:1,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours.

The immunogenic composition can further include excipients or diluents that are pharmaceutically acceptable as carriers and compatible with the immunogenic composition. The term "pharmaceutically acceptable carrier" refers to a carrier(s) that is "acceptable" in the sense of being compatible with the other

ingredients of a composition and not deleterious to the recipient thereof. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the immunogenic composition may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the immune-stimulating composition.

The immunogenic composition can be used in a method for protecting an animal from a disease caused by *P. gingivalis*. This method includes administering the immunogenic composition and eliciting antibodies to a prolyl peptidase, antigenic analog, antigenic fragment, or antigenic modification. The diseases that can be treated in this manner include periodontal diseases, which includes gingivitis and periodontitis. Clinical hallmarks of periodontitis include lose of tooth attachment and periodontal pocket formation.

Alternatively and preferably, periodontal diseases can be treated by the use of inhibitors of a prolyl peptidase. An inhibitor of a prolyl peptidase, preferably a prolyl tripeptidyl-peptidase, can be present in a composition that preferably contains a pharmaceutically acceptable carrier. For instance, inhibitors can be applied systemically, subgingivally (e.g., subgingival irrigation) and/or by controlled release delivery directly into the periodontal pocket using methods well known to the art (see, e.g., Kornman, K., (1993) *J. Periodontol.* 64, 782-791). Preferably, an inhibitor is applied subgingivally or by controlled release delivery.

The prolyl peptidases, active analogs, fragments, and modifications thereof can be used in a method of reducing growth of bacteria *in vitro* or *in vivo*. Preferably, the bacteria is a periodontal pathogen, i.e, a bacterial pathogen that causes periodontal disease, more preferably the bacteria is *P. gingivalis*. The inability of asaccharolytic *P. gingivalis* to utilize free amino acids makes the bacterium entirely dependant on an external peptide supply. The action of the polypeptides of the invention may be required for bacterial growth, and inhibition of the polypeptides of the invention may inhibit the *in vivo* growth of organisms, including *P. gingivalis*. The method includes decreasing the amount of dipeptides and/or tripeptides (e.g., the result of cleavage of SEQ ID NO:25 by a prolyl-tripeptidyl peptidase) and the amount of free amino acids that result from further cleavage of the dipeptides and/or tripeptides present by inhibiting a prolyl

peptidase, active analog, active fragment, or active modification thereof, such that the amount of dipeptides and/or tripeptides generated by the polypeptides is decreased. The amount of dipeptides and/or tripeptides is decreased relative to the amount of dipeptides and/or tripeptides present in the absence of the inhibitor. Preferably, the amount of dipeptides and/or tripeptides generated is decreased by an inhibitor, a monoclonal antibody that inhibits the prolyl peptidase, or polyclonal antibodies that inhibit the prolyl peptidase, more preferably, the amount of dipeptides and/or tripeptides generated is decreased by an inhibitor. Preferably, an inhibitor acts to inhibit a polypeptide of the invention, preferably a prolyl peptidase, by blocking the active site of the polypeptide. The polypeptide can be present on the surface of the bacteria or secreted into the environment, preferably the polypeptide is present in the surface of the bacteria.

The present invention is also directed to a method of developing an inhibitor of a prolyl peptidase, active analog, active fragment, or active modification thereof, preferably a prolyl-tripeptidyl peptidase. The method includes identifying a molecule that inhibits the amidolytic activity of the prolyl peptidase. This can be accomplished by, for instance, incubating the prolyl peptidase with a candidate molecule under conditions that promote amidolytic activity of the prolyl peptidase and determining if the amidolytic activity of the prolyl peptidase is decreased relative to the amidolytic activity in the absence of the molecule. The amidolytic activity can be measured by cleavage of the Pro-Yaa peptide bond present in the target polypeptide SEQ ID NO:25 as described herein. One method of developing an inhibitor includes using the target peptide SEQ ID NO:25 and replacing the Xaa residues with modified amino acids. It is expected that some modified amino acids will cause the target peptide to act as an inhibitor.

EXAMPLES

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

Example 1

Materials

Diisopropylfluorophosphate (DFP), leupeptin and 3,4-dichloroisocoumarin, were purchased from Calbiochem (La Jolla, CA). Antipain, iodoacetamide, substance P, bradykinin and bradykinin related peptides, were obtained from Sigma. Other peptides used in this study were synthesized at the Molecular Genetic Instrumental Facility (University of Georgia, Athens, GA) using Fmoc protocol with an advanced ChemTech MPS350 automated synthesizer. H-Ala-Phe-Pro-pNA, H-Gly-Pro-pNA, Z-Gly-Pro-pNA, Z-Ala-Pro-pNA, and H-Pro-pNA (where pNA is p- Nitroanilide; Z is benzyloxycarbonyl; and H is hydrogen and denotes an unblocked amino-terminal group) were obtained from Bachem (King of Prussia, PA). Prolinal was kindly provided by Dr. James Powers (Georgia Institute of Technology, Atlanta) and cystatin C by Dr. Magnus Abrahamson (University of Lund, Sweden).

Methods

Source and Cultivation of Bacteria— *P. gingivalis* HG66 was obtained from Dr. Roland Arnold (University of North Carolina, Chapel Hill), while the strains W50 (ATCC 53978) and ATCC 33277 were obtained from the ATCC. All cells were grown as described previously (Chen, Z., et al., (1992) *J. Biol. Chem.* 267, 18896-18901).

Enzyme Activity Assays— Routinely, the tripeptidyl peptidase amidolytic activity was measured with H-Ala-Phe-Pro-pNA (1mM) in 0.2 M HEPES (N-2-hydroxyethylpeperazine,N'-2-ethansulfonic acid), pH 7.5 at 37°C. The concentration of enzyme was 0.1 nM to 1 nM. The assay was performed in a total volume of 0.2 ml on microplates, and the initial turnover rate was recorded at 405 nm using a microplate reader (Spectramax Molecular Devices, Sunnyvale, CA). In inhibition studies, the enzyme was first preincubated with inhibitor for 15 min at 37°C, substrate added, and residual activity recorded after 5 minutes to 30 minutes. H-Gly-Pro-pNA, Z-Ala-Pro-pNA, Z-Gly-Pro-pNA and H-Pro-pNA (1 mM final concentration) were assayed in the same manner.

Protein Determination—Protein concentration was determined using the BCA reagent kit (Sigma, St. Louis, MO), using bovine serum albumin as a standard.

Localization of Tripeptidyl-Peptidase Activity—Cultures of *P. gingivalis* HG66, W50 and ATCC 33277, at different phases of growth, were subjected to the following fractionation procedure. The cells were removed by centrifugation (10,000 x g, 30 minutes), washed twice with 10 mM Tris, 150 mM NaCl, pH 7.4, resuspended in 50 mM Tris, pH 7.6, and disintegrated by ultrasonication in an ice bath at 1500 Hz for 5 cycles (5 minutes sonication/5 minutes brake). Unbroken cells and large debris were removed by centrifugation (10,000 x g, 30 minutes) and the opalescent supernatant subjected to ultracentrifugation (150,000 x g, 120 minutes), yielding a pellet containing bacterial membranes and a supernatant which was considered as membrane-free cell extract. All fractions, as well as the full culture, culture medium, and full culture after sonication, were assayed for amidolytic activity against H-Ala-Phe-Pro-pNA.

Enzyme Purification—All purification steps were performed at 4°C except for FPLC separations, which were carried out at room temperature. Cells were harvested by centrifugation (6,000 x g, 30 minutes), washed with 50 mM potassium phosphate buffer, pH 7.4, and resuspended in the same buffer (150 ml per 50 gram of cells wet weight). Triton X-100 (10% volume/volume in H_2O) was added slowly to the bacterial cell suspension to a final concentration of 0.05%. After 120 minutes of gentle stirring, unbroken cells were removed by centrifugation (28,000 x g, 60 minutes). Proteins in the supernatant were precipitated with cold acetone (-20°C) added to a final concentration of 60% and collected by centrifugation. The pellet was redissolved in 50 mM potassium phosphate buffer, pH 7.0, and extensively dialyzed against 20 mM potassium phosphate, pH 7.0, containing 0.02% sodium azide. The dialyzed fraction was clarified by centrifugation (28,000 x g, 30 min) and applied to a hydroxyapatite column (BioRad, Melville, NY) equilibrated with 20 mM potassium phosphate, pH 7.0, at a flow rate of 20 ml/hour. After equilibration, the column was washed until the A_{280} fell to zero. Bound proteins were eluted with a gradient from 20-300 mM potassium phosphate and fractions (7 ml) analyzed for dipeptidyl- and tripeptidyl-peptidase activity using H-Gly-Pro-pNA and H-Ala-Phe-Pro-pNA, respectively. The activity against the latter

substrate was pooled, saturated with 1 M ammonium sulfate, clarified by centrifugation, and directly loaded onto a Phenyl-Sepharose HP (Pharmacia, Piscataway, NJ) column equilibrated with 50 mM potassium phosphate, pH 7.0, containing 1 M ammonium sulfate. The column was washed with two volumes of equilibration buffer, followed by buffer containing 0.5 M ammonium sulfate, and developed with a descending gradient of ammonium sulfate from 0.5 to 0 M. Active fractions were pooled, extensively dialyzed against 20 mM Tris, pH 7.5, and applied to a MonoQ HR 5/5 FPLC column equilibrated with the same buffer. The column was washed with 5 volumes of equilibration buffer at 1.0 ml/minute, following which bound proteins were eluted with a gradient of 0- 300 mM NaCl. The active fractions were pooled, dialyzed against 25 mM Bis-Tris, pH 6.3, and subjected to chromatofocusing on a MonoP FPLC column equilibrated with Bis-Tris buffer, using a pH gradient developed with 50 ml of 10x diluted Polybuffer 74 (Pharmacia), adjusted to a pH of 4.0.

Electrophoretic Techniques— The SDS-PAGE system of Schagger and von Jagow (Schagger, H., and von Jagow, G. (1987) *Anal. Biochem.* 166, 368-379), was used to monitor enzyme purification and estimate the enzyme molecular mass. For amino-terminal sequence analysis, proteins resolved in SDS-PAGE were electroblotted to polyvinylidene difluoride membranes using 10 mM CAPS, pH 11, 10% methanol (Matsudaira, P. (1987) *J. Biol. Chem.* 262, 10035-10038). The membrane was washed thoroughly with water and stained with Coomasie Blue G250. The blot was air dried, and protein bands cut out and subjected to NH_2-terminal sequence analysis with an Applied Biosystems 491 Protein Sequencer using the program designed by the manufacturers.

Enzyme Fragmentation —The purified prolyl tripeptidyl peptidase (PTP-A) was partially denatured by incubation in 6 M urea in 0.02 M Tris, pH 7.6, for 60 minutes. Low molecular mass gingipain R (RgpB) (Potempa, J., et al. (1995) *Prospect. Drug Discovery and Design* 2, 445-458) from *P. gingivalis* was then added to make an enzyme:substrate molar ratio of 1:100. The reaction mixture was made in 1 mM cysteine and the sample incubated overnight at 37°C. Generated peptides were separated by reverse-phase HPLC using a μBondapak C-18 column (3.9 x 300 mm) (Waters, Millford, MA). Peptides were eluted with 0.1% trifluoroacetic acid and acetonitrile containing 0.08% trifluoroacetic acid, using a

gradient from 0 to 80% acetonitrile over 60 minutes. Peptides were monitored at 220 nm and collected manually.

For determination of the active site serine residue and to confirm that the purified enzyme was a serine peptidase, 100 μg of purified PTP-A was first incubated with 170 μCi of [1,3-^{3}H]DFP (Amersham, Arlington Heights, IL) for 30 minutes at 25°C in 20 mM HEPES, pH 7.5. The reaction was quenched by addition of cold DFP to a final concentration of 10 mM and the radiolabelled material analyzed by SDS-PAGE, followed by autoradiographic analysis. The gel was dehydrated, soaked in PPO solution for 2 hours, dried, and the DFP-binding proteins detected by fluorography after an exposure time of 96 hours on X-ray film (XAR; Kodak, Rochester, NY). The bulk of radiolabelled protein was subjected to proteolytic fragmentation with RgpB and peptides obtained separated by reverse-phase HPLC as described above. Radioactivity in each peptide fraction was measured using a β liquid scintillation counter, and the labeled peptide, as well as other selected peptides were subjected to sequence analysis.

Identification of the PTP-A Gene— The database containing the unfinished *P. gingivalis* W83 genome, available from The Institute for Genomic Research, was searched for the presence of nucleotide sequences corresponding to the NH_2-terminal and the internal PTP-A amino acid sequences using the TBLASTN algorithm, BLAST version 2.0.8, and the default values for all parameters (Altschul, S.F., et al.,(1997) *Nucleic Acid Res.* 25, 3389-3402). An identified clone gnl | TIGR | P. gingivalis_126 was retrieved from The Institute for Genomic Research data base (http://www.tigr.org). The position of the PTP-A gene was localized using the NCBI open reading frame (ORF) finder (available from the National Center for Biotechnology Information, at http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The amino acid sequence, obtained by conceptual translation of the entire ORF, was further used for homology screening by use of the NCBI BLAST search tool.

Enzyme Specificity— Peptides were incubated with 1 μg PTP-A at an enzyme:substrate molar ratio of 1:100 for 3 hours or 24 hours in 50 μl of 200 mM HEPES, pH 7.5, at 37°C, and the reaction stopped by acidification with trifluoroacetic acid. The samples were then subjected to reverse-phase high

pressure liquid chromatography using a μBondapak C-18 column (3.9 x 300 mm) (Waters, Millford, MA) and an acetonitrile gradient (0-80 % in 0.075% trifluoroacetic acid in 50 min). Each peak, detected at 220 nm, was collected, lyophilized, re-dissolved in 50% (volume/volume) methanol, 0.1% acetic acid and subjected to analysis by mass spectrometry.

Mass Spectrometry—A Finnigan MAT 95S, sector mass spectrometer (Finnigan MAT, Bremen, Germany) equipped with an electrospray source (ESI) was used operated essentially as described previously (Stenfors, C., et al., (1997) *J. Biol. Chem.* 272, 5747-5751). Peptides were identified by fitting of the obtained spectra to specific sequences using an Internet application program MsFit available at http://falcon.ludwig.ucl.ac.uk/msfit.html.

Example 2

Enzyme Localization, Purification and Initial Characterization

Analysis of amidolytic activity against H-Ala-Phe-Pro-pNA in several fractions of *P. gingivalis* HG66, W50 and ATCC 33277 clearly indicated that an enzyme(s) with prolyl tripeptidyl-peptidase activity is localized on the cell surface in all strains tested with less than 5% of the total activity being found in the medium regardless of the growth phase of the bacterial culture. Cell associated enzyme was easily detached from the bacterial surface by treatment with a low concentration (0.05%) of Triton X-100. This procedure released more than 85-90% of activity in a soluble form. Subsequent acetone precipitation of proteins in the Triton X-100 fraction successfully separated the activity from pigment which remained in solution. The redissolved protein fraction, after dialysis, was applied to hydroxyapatite (100 ml) equilibrated with 20 mM potassium phosphate buffer pH 7.0. The elution was carried out with 20 mM potassium phosphate buffer pH 7.0, using a phosphate gradient from 20 mM to 300 mM at flow rate 20 ml/h. At this step substantial separation of the PTP-A activity from both the DPP IV and bulk protein was achieved (Fig. 1a). Further purification performed by subsequent chromatography steps including Phenyl-Sepharose (Fig. 1b), MonoQ (Fig. 1c) and MonoP columns (Fig. 1d), resulted in the isolation of purified enzyme.

Phenyl-Sepharose HP (25 ml) was equilibrated with 50 mM potassium phosphate, 1M ammonium sulfate, pH 7.0, at flow rate 30 ml/h. The column was

washed with two volumes of equilibration buffer and a step gradient of 0.5 M ammonium sulfate was applied, following which a descending gradient of 0.5 to 0 M ammonium sulfate was applied. The PTP-A containing fractions were extensively dialyzed against 20 mM Tris-HCl, pH 7.0, and concentrated by ultrafiltration. The concentrated PTP-A containing fractions were applied to a MonoQ column equilibrated with the same buffer. The column was washed with 5 volumes of equilibration buffer, following which bound protein was eluted with a gradient of 0-300 mM NaCl. The concentrated fraction of PTP-A from the MonoQ column was equilibrated with 25 mM Bis-Tris, pH 6.3, and loaded on a MonoP column equilibrated with the same buffer. A pH gradient was developed using 50 ml of Polybuffer 74, with the pH adjusted to 4.0.

Significantly, the chromatography step on the MonoP column yielded the A_{280} profile much sharper than the activity peak. Although this imperfect overlap of protein and activity may suggest that the protein component does not represent the active enzyme, the rest of data argues with such a contention. This apparent contradiction may be likely explained by the enzyme inhibition by the reaction product of H-Ala-Phe-Pro-pNA hydrolysis but this possibility has not been explored. The yield of protein and activity recovery in a typical purification procedure is summarized in Table 1.

Table 1. Purification of the PTP- A from *P. gingivalis*

Step	Volume (ml)	Protein (mg)	Total activity*	Specific activity (units/mg)	Purification fold	Yield (%)
Triton X-100 extract after centrifugation	200	1200	757 673	642	1	100
Acetone precipitate	50	600	537 622	896	1.4	71
Hydroxyapatite chromatography	50	22	400 039	18 183	28	53
Phenyl-Sepharose	48	10	312 505	31 250	48	41
MonoQ	3	1.5	244 828	163 218	254	32
MonoP	4	0.7	188 400	269 142	420	25

* Based on the enzymatic activity using H-Ala-Phe-Pro-pNA where one unit = mOD/min/1ml

SDS-PAGE analysis of the purified enzyme revealed the presence of two protein bands with apparent molecular masses of 81.8 and 75.8 kDa, respectively (Fig. 2, lane f). Autoradiography of the enzyme sample radiolabeled with [1,3-^{3}H]DFP (Fig. 2, lane g) clearly indicated that the bands represented either two distinct serine peptidases or different molecular mass forms of the same enzyme. In an attempt to distinguish between these two options, the electrophoretically resolved proteins were subjected to amino terminal sequence analysis. Unfortunately, it was found that the 81.8 kDa form of PTP-A had a blocked N-terminus. In contrast, the sequence NH_2-SAQTTRFSAADLNALMP (SEQ ID NO:23) was found at the N-terminus of the lower molecular mass form of the enzyme. This result led us to the possibility that the 75.8 kDa form of PTP-A was derived from the 81.8 kDa form through proteolytic cleavage of a 6 kDa amino-terminal peptide. To confirm this hypothesis and, in addition, to localize the active site residue within *P. gingivalis* PTP-A, the mixture containing both radiolabeled enzymes was proteolytically fragmented and peptides resolved by reverse-phase HPLC. This procedure yielded only one major radioactive peptide peak, and the purified peptide was found to have a single amino acid sequence: IGVHGWXYGGFMTTNL (SEQ ID NO:24), where X apparently represents the active-site serine residue covalently and irreversibly modified by DFP. These data convincingly indicate that the two protein bands of purified PTP-A represents different forms of the same enzyme. The portion of the purified PTP-A having a truncated N-terminus may be due to cleavage by Lys-specific peptidase and is likely to be an artifact which occurred during the purification procedure. Nevertheless, the proteolytic shedding of membrane bound PTP-A also occurs during cultivation of the bacteria, as indicated by variable amount of soluble activities found in cell free culture media.

Example 3

pH Optimum, Stability and Inhibition Profile

Using the amidolytic activity assay with H-Ala-Phe-Pro-pNA it was found that the enzyme has a broad pH optimum from pH 6.0 to 8.0 and in 0.2 M HEPES, pH 7.6 was stable for at least 12 hours at 25°C or 37°C. PTP-A activity was not affected by class specific synthetic inhibitors of cysteine or metalloproteinases

(Table 2). In contrast, preincubation of the enzyme with DFP or PEFABLOCK resulted in total loss of activity, supporting its classification as a serine peptidase. Surprisingly, however, 3,4-dichloroisocumarin was only a poor inhibitor, and PMSF, leupeptin, antipain and prolinal had no effect at all. Interestingly, preincubation of PTP-A with iodoacetamide, but not with N-ethylmaleimide, stimulated enzyme amidolytic activity about two-fold. Human plasma inhibitors, such as α_1-proteinase inhibitor, α_1-antichymotrypsin and α_2-macroglobulin did not affect the enzyme activity, nor were they cleaved by PTP-A.

The effect of inhibitors on amidolytic activity of DPP IV was also determined using the same conditions as those used for PTP-A, but using H-Gly-Pro-pNA as a substrate.

Table 2. Effect of inhibitors on the amidolytic activity of PTP-A and DPP IV. Results are for a 15-min incubation at 37 C in 0.2 HEPES pH 7.6, with 1 mM H-Ala-Phe-Pro-pNA as substrate.

Inhibitor	Concentration	Residual activity of PTP-A, %	Residual activity of DPP IV, %
Diisopropyl fluorophospate	10 mM	0	0
	10 mM	96	20
Phenylmethanesulfonyl fluoride	1mg/ml	20	15
	10mg/ml	0	0
PEFABLOC SC	1 mM	56	100
3,4-dichloroisocoumarin	5mM	200	100
Iodoacetamide	5 mM	100	100
N-Ethylmaleimide	1 mM	98	100
1,10-orthophenanthroline	5 mM	93	100
	0.1 mM	100	100
EDTA	0.1 mM	100	100
Leupeptin	0.1 mM	100	20
Antipain	10 mM	100	0
Prolinal	10 mM	100	30
Val-Pro	10 mM	100	1
Ala-Pro			
Ala-Gly-Pro			

Example 4

Substrate Specificity

Among several chromogenic substrates tested, including H-Ala-Phe-Pro-pNA, H-Gly-Pro-pNA, Z-Gly-Pro-pNA, Z-Ala-Pro-pNA, H-Pro-pNA, only H-Ala-Phe-Pro-pNA was hydrolyzed by PTP-A indicating a prolyl specific tripeptidyl-peptidase activity. To further confirm this specificity several synthetic peptides composed of 5 to 34 amino acid residues and containing at least one proline residue were tested as substrates for PTP-A. Out of 22 peptides tested only those with a proline residue in the third position from the amino terminal end were cleaved (Table 3), with the significant exception of peptides with adjacent proline residues (peptides 3, 4 and 16). In addition, a free α-amino group was absolutely required for cleavage after the third proline residue as exemplified by resistance to enzymatic hydrolysis of peptide 9, which differs from the peptide 8 only in acylation of the α-amino group of the N-terminal valine residue. Except for these two limitations, the peptide bond -Pro-↓-Yaa- was cleaved at the same rate in all peptides with the general formula NH_2-Xaa-Xaa-Pro-Yaa-$(Xaa)_n$ (SEQ ID NO:25), where Xaa represents any amino acid residue while Yaa could be any residue except proline, regardless of the chemical nature of the amino acids and the length of the peptide. In all cases the reaction was completed within 3 hours and prolonged incubation for 24 hours did not affect the pattern of cleavage, confirming the absolute requirement for a proline residue at the third position from the unblocked N-terminus. In addition, these data indicate that the preparation of PTP-A was free of any contamination with either aminopeptidase, dipeptidyl peptidase, or endopeptidase activities.

The cleavage specificity of DPP IV was also determined using the same conditions as those used for PTP-A. The results (Table 3) demonstrate that DPP IV does not cleave between two proline residues.

Table 3. Cleavage specificity of PTP-A and DPP IVon synthetic peptides.

Substrate	Cleavage site	SEQ ID NO:
Peptide 1	H-Arg-Pro-**Pro**-↓-Gly-Phe-Ser-**Pro**-Phe-Arg	1
Peptide 2	H-Arg-**Pro**-**Pro**-↓-Gly-Phe	2
Peptide 3	H-Lys-Arg-**Pro**-**Pro**-Gly-Phe-Ser-**Pro**-Phe-Arg	3
Peptide 4	H-Tyr-Arg-**Pro**-**Pro**-Gly-Phe-Ser-**Pro**-Phe-Arg	4
Peptide 5	H-Arg-**Pro**-**Hyp**-Gly-Phe-Ser-**Pro**-Phe-Arg	5
Peptide 6	H-Arg-**Pro**-↑-Lys-**Pro**-↑-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2	6
Peptide 7	H-Val-**Pro**-**Pro**-↓-Gly-Glu-Asp-Ser-Lys-Glu-Val-Ala-Ala-**Pro**-His-Arg-Gln	7
Peptide 8	H-Val-**Pro**-**Pro**-↓-Gly-Glu-Asp-Ser-Lys	8
Peptide 9	Ac-Val-**Pro**-**Pro**-Gly-Glu-Asp-Ser-Lys	9
Peptide 10	H-Val-Glu-**Pro**-↓-Ile-**Pro**-Tyr	10
Peptide 11	H-Arg-Gly-**Pro**-↓-Phe-**Pro**-Ile	11
Peptide 12	H-Ala-Arg-**Pro**-↓-Ala-D-Lys-amide	
Peptide 13	H-**Pro**-Asn-**Pro**-↓-**Asn**-Gln-Gly-Asn-Phe-Ile	13
Peptide 14	H-Arg-His-**Pro**-↓-Lys-Tyr-Lys-Thr-Glu-Leu	14
Peptide 15	H-Gly-Val-**Pro**-↓-Lys-Thr-His-Leu-Glu-Leu	15
Peptide 16	H-Lys-Gly-**Pro**-**Pro**-Ala-Ala-Leu-Thr-Leu	16
Peptide 17	H-Gln-Lys-Gln-Met-Ser-Asp-Arg-Arg-Glu-Asn-Asp-Met-Ser-**Pro**-Ser-Asn-Asn-Val-Val-**Pro**-Ile-His-Val-**Pro**-**Pro**-Thr-Thr-Glu-Asn-Lys-**Pro**-Lys-Val-Gln	17
Peptide 18	H-Phe-Leu-Arg-Glu-**Pro**-Val-Ile-Phe-Leu	18
Peptide 19	H-Gly-Ile-Arg-**Pro**-Tyr-Glu-Ile-Leu-Ala	19
Peptide 20	H-Leu-**Pro**-↑-Asp-Leu-Asp-Ser-Ser-Leu-Ala-Ser-Ile-Gln-Glu-Leu-Leu-Ser-**Pro**-Gln-Glu-**Pro**--**Pro**-Arg-**Pro**-**Pro**-Glu-Ala	20
Peptide 21	H-Cys-Leu-Ser-Ser-Gly-Thr-Leu-**Pro**-Gly-**Pro**-Gly-Asn-Asp-Ala-Ser-Arg-Glu-Leu-Glu-Ser	21
Peptide 22	H-Lys-Ile-**Ala**-Gly-Tyr-His-Leu-Glu-Leu	22
Peptide 23	H-Ser-**Pro**-↑-Tyr-Ser-Ser-Asp-Thr-Thr	46
Peptide 24	H-Ala-**Pro**-↑-Val-Arg-Ser-Leu-Asn-Cys-Thr-Leu-Arg-Asp-Ser-Gln-Gln-Lys	47

↓ indicates cleavage site mediated by PTP-A

↑ indicates cleavage site mediated by DPP IV

The lack of cleavage after internal proline residues in the synthetic peptides corresponds well with the absence of any proteolytic activity on several protein substrates including IgA, IgG, albumin, azocasein, carboxymethylated rybonuclease and gelatin. However, the size of substrate, which is a limiting factor in the activity of oligopeptidases (Walter, R., et al., (1980) *Mol. Cell. Biochem.* 30, 111-126), is not restricting in the case of PTP-A, because the enzyme is able to cleave a tripeptide (NH_2-Xaa-Xaa-Pro) from the N-terminus of both human cystatin C and interleukin 6.

Example 5

PTP-A Sequence Analysis

Partial PTP-A amino acid sequence data allowed us to identify the *P. gingivalis* genomic clone gnl | TIGR | P. gingivalis_126 in the Unfinished Microbial Genomes data base, TIGR. An ORF corresponding to the PTP-A amino acid sequence was found as indicated by the fact that all sequences of the PTP-A derived peptides obtained by the enzyme polypeptide fragmentation with RgpB were present in the protein primary structure inferred from the nucleotide sequence of the ORF. The 732 amino acid polypeptide with a calculated mass of 82, 266 Da was encoded in this ORF. The homology search performed using the NCBI TBLASTN tool against GenBank+EMBL+DDBJ+PDB databases and subsequent multiple sequence alignments using the ClustalW Multiple Sequence Alignment tool (Fig. 3) indicated that PTP-A is a new member of the prolyl oligopeptidase (S9) family of serine peptidases (Rawlings, N.D., et al., (1991) *Biochem. J.* 279, 907-908).

The sequence GXSXGG (SEQ ID NO:40) is a signature feature for the S9 family of serine peptidases. Within this large and diverse S9 family of evolutionary and functionally related enzymes both from prokaryotes and eukaryotes, PTP-A was most closely related to bacterial dipeptidyl peptidase IV (DPP IV) from *Flavobacterium meningosepticum*, *Xantomonas maltophilus*, and *P. gingivalis*, sharing 31.6%, 30.4%, and 28.5% amino acid sequence identity, respectively. Remarkably, the COOH-terminal region of the PTP-A molecule (residues 502 - 732) shows a significant similarity to the eukaryotic prolyl oligopeptidases with 34% and 33% identity to human DPP IV and mouse fibroblast activation protein (FAP), respectively (Fig. 3). This part of the molecule contains the amino acid

residues which encompass the catalytic triad in all characterized prolyl oligopeptidases, and from the multiple alignments with DPP IV of confirmed active site residues (Kabashima,T., et al., (1995) *Arch. Biochem. Biophys.* 320, 123-128) it is apparent that Ser-603, Asp-678 and His-710 represent the catalytic triad of PTP-A (Fig. 3). Such an inference is further supported by the direct labeling of Ser-603 by DFP. In addition, the computer assisted search for sequential motifs characteristic for transmembrane domains revealed the presence of such a putative region within the N-terminal sequences of PTP-A, with residues 5 to 25 most likely folded into a hydrophobic α-helix responsible for membrane anchoring of this enzyme.

In *P. gingivalis* PTP-A, as well as in DPP IV, all activities are cell surface associated, and it is conceivable that the enzymes are membrane anchored through putative signal sequences which are not cleaved but remain as a membrane spanning domain similar to other members of the prolyl oligopeptidase family. The cell surface localization of di- and tripeptidyl-peptidases suggests a putative physiological function in providing nutrients for growing bacterial cells. Here, the inability of asaccharolytic *P. gingivalis* to utilize free amino acids (Dashper, S.G., et al., *J. Dent Res.* 77 ,1133 (Abstract) (1988)) makes the bacterium entirely dependant on an external peptide supply. In this regard, DPP-IV and PTP-A activities are probably very important, if not indispensable, for bacterial growth.

This suggestion is strongly corroborated by the fact that the *P. gingivalis* genome contains three additional genes encoding peptidases homologous with DPP-IV and PTP-A and one related to aminopeptidase B. The peptidases homologous with DPP-IV and PTP-A are referred to as homologs H1 (SEQ ID NO:43), H2 (SEQ ID NO:44), and H3 (SEQ ID NO:45) (Fig. 6). If expressed, each gene product would probably have enzymatic activity because each has a well preserved catalytic triad (Fig. 4). In addition, all of these genes encode a putative signal peptide which may act in providing membrane-anchorage motifs.

Example 6

Influence of Proteinase Inhibitor on *P. gingivalis* Growth

To evaluate whether *P. gingivalis* growth was influenced by the presence of a peptidase inhibitor, *P. gingivalis* in logphase growth was diluted 1:5 into liquid media and incubated at 37°C. The cell density was monitored by measuring the optical density at 600 nm (OD_{600}). When the optical density began to increase, Pefabloc was added at 0.5 mg/ml or at 2.0 mg/ml. The control culture received no Pefabloc. The cultures receiving Pefabloc exhibited decreased growth (Figure 5). The peptidase inhibitor had to be added before the culture reached an OD_{600} of about 0.3 for the peptidase inhibitor to have an effect on growth.

The complete disclosures of all patents, patent applications, publications, and nucleic acid and protein database entries, including for example GenBank accession numbers and EMBL accession numbers, that are cited herein are hereby incorporated by reference as if individually incorporated. Various modifications and alterations of this invention will become apparent to those skilled in the art without departing from the scope and spirit of this invention, and it should be understood that this invention is not to be unduly limited to the illustrative embodiments set forth herein.

Sequence Listing Free Text

SEQ ID NOs:1-11:	Synthetic peptides
SEQ ID NO:12:	Target peptide
SEQ ID NOs:13-22:	Synthetic peptides
SEQ ID NO:23:	Amino-terminus of the lower molecular mass form of PTP-A.
SEQ ID NO:24:	Amino acid sequence present in PTP-A, where X apparently represents the active-site serine residue covalently and irreversibly modified by DFP.
SEQ ID NO:25:	Target peptide, where Xaa represents a natural or modified amino acid residue, Yaa represents a natural or modified amino acid residue except proline, and N is equal to or greater than 1.
SEQ ID NO:26:	Mouse fibroblast activation protein

SEQ ID NO:27:	Human DPP IV
SEQ ID NO:28:	DPP from *Flavobacterium meningosepticum*
SEQ ID NO:29:	DPP from *P. gingivalis*
SEQ ID NO:30:	*P. gingivalis* PTP-A
SEQ ID NO:31:	Portion of PTP-A
SEQ ID NO:32:	Portion of DPP from *P. gingivalis*
SEQ ID NO:33:	Portion of H1 homolog of *P. gingivalis* DPP
SEQ ID NO:34:	Portion of H2 homolog of *P. gingivalis* DPP
SEQ ID NO:35:	Portion of H3 homolog of *P. gingivalis* DPP
SEQ ID NOs:36-37:	Probes
SEQ ID NO:38:	Nucleotide sequence of coding region encoding PTP-A.
SEQ ID NO:39:	Consensus sequence for clan SC where X is any amino acid and S is the active site serine GXSXXG.
SEQ ID NO:40:	Consensus sequence for family S9 where X is any amino acid and S is the active site serine GXSXGG.
SEQ ID NO:41:	A specific substrate for a prolyl-tripeptidyl peptidase, where Xaa represents a natural or modified amino acid residue, and Yaa represents a natural or modified amino acid residue except proline.
SEQ ID NO:42:	DPP from *P. gingivalis*
SEQ ID NO:43:	H1 homolog of *P. gingivalis* DPP
SEQ ID NO:44:	H2 homolog of *P. gingivalis* DPP
SEQ ID NO:45:	H3 homolog of *P. gingivalis* DPP
SEQ ID NO:46:	Synthetic peptides
SEQ ID NO:47:	Synthetic peptides
SEQ ID NO:48:	Amino terminal sequence of DPP IV

What is claimed is:

1. An isolated prolyl tripeptidyl-peptidase, active analog, active fragment, or active modification thereof having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids, wherein the prolyl tripeptidyl-peptidase:target polypeptide ratio is at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours.

2. An isolated prolyl tripeptidyl-peptidase, active analog, active fragment, or active modification thereof having amidolytic activity at a prolyl tripeptidyl-peptidase:target polypeptide ratio of at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours, and wherein the prolyl tripeptidyl-peptidase is isolated from *P. gingivalis*.

3. The isolated prolyl tripeptidyl-peptidase of claim 2 wherein the peptide cleaved by the isolated prolyl tripeptidyl-peptidase comprises the sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:11, H-Ala-Arg-Pro-Ala-D-Lys-amide, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:25, and SEQ ID NO:37.

4. The isolated prolyl tripeptidyl-peptidase of claim 2 wherein the amino acid sequence of the isolated prolyl tripeptidyl-peptidase comprises the amino acid sequence GXSXXG (SEQ ID NO:39).

5. The isolated prolyl tripeptidyl-peptidase of claim 4 wherein the amino acid sequence of the isolated prolyl tripeptidyl-peptidase comprises the amino acid sequence GXSXGG (SEQ ID NO:40).

6. The isolated prolyl tripeptidyl-peptidase of claim 4 wherein the amino acid sequence of the isolated prolyl tripeptidyl-peptidase comprises SEQ ID NO:30.

7. An isolated polypeptide, active analog, active fragment, or active modification thereof having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids, wherein the polypeptide:target polypeptide ratio of at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours.

8. An isolated polypeptide comprising an amino acid sequence having a percentage amino acid identity of greater than 35 % with SEQ ID NO:30.

9. An isolated nucleic acid fragment encoding a prolyl-tripeptidyl peptidase, active analog, active fragment, or active modification thereof, having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids, wherein the prolyl tripeptidyl-peptidase:target polypeptide ratio of at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours.

10. The nucleic acid fragment of claim 9 wherein the nucleic acid fragment has a nucleotide sequence comprising SEQ ID NO:38.

11. The nucleic acid fragment of claim 9 wherein a complement of the nucleic acid fragment hybridizes to SEQ ID NO:38 under hybridization conditions of 0.5 M phosphate buffer, pH 7.2, 7 % SDS, 10 mM EDTA, at 68°C, followed by three for 20 minutes washes in 2x SSC, and 0.1 % SDS, at 65°C, wherein at least about 20 nucleotides of the complement hybridize.

12. An isolated nucleic acid fragment encoding a polypeptide wherein the polypeptide comprises an amino acid sequence having a percentage amino acid identity of greater than 35 % with SEQ ID NO:30.

13. A method of identifying an inhibitor of a prolyl-tripeptidyl peptidase, active analog, active fragment, or active modification thereof, comprising identifying a molecule that inhibits the amidolytic activity of the prolyl-

tripeptidyl peptidase by incubating the prolyl-tripeptidyl peptidase with the molecule under conditions that promote amidolytic activity of the prolyl-tripeptidyl peptidase and determining if the amidolytic activity of the prolyl-tripeptidyl peptidase is inhibited relative to the amidolytic activity in the absence of molecule.

14. A method of reducing growth of a bacterium comprising inhibiting a prolyl tripeptidyl-peptidase, active analog, active fragment, or active modification thereof, by contacting the prolyl tripeptidyl-peptidase with an inhibitor of the prolyl tripeptidyl-peptidase.

15. A method for protecting an animal from a periodontal disease caused by *P. gingivalis* comprising administering to the animal the inhibitor of claim 14 wherein the disease is selected from the group consisting of gingivitis and periodontitis.

16. The method of claim 15 wherein the inhibitor is administered by a method selected from the group consisting of subgingival application and controlled release delivery.

17. A method of reducing growth of a bacterium comprising inhibiting a prolyl dipeptidyl-peptidase, active analog, active fragment, or active modification thereof, by contacting the prolyl dipeptidyl-peptidase with an inhibitor of the prolyl dipeptidyl-peptidase.

18. An immunogenic composition comprising an isolated prolyl tripeptidyl-peptidase, or an antigenic analog, antigenic fragment, or antigenic modification thereof, the prolyl tripeptidyl-peptidase having amidolytic activity for cleavage of a peptide bond present in a target peptide having at least 4 amino acids, wherein the prolyl tripeptidyl-peptidase:target polypeptide ratio is at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours.

19. The immunogenic composition of claim 18 further comprising an adjuvant.

20. A composition comprising an inhibitor of an isolated prolyl tripeptidyl-peptidase and a pharmaceutically acceptable carrier.

21. A dipeptidyl peptidase having an amino acid sequence comprising SEQ ID NO:43.

22. A dipeptidyl peptidase having an amino acid sequence comprising SEQ ID NO:44.

23. A dipeptidyl peptidase having an amino acid sequence comprising SEQ ID NO:45.

Fig 1

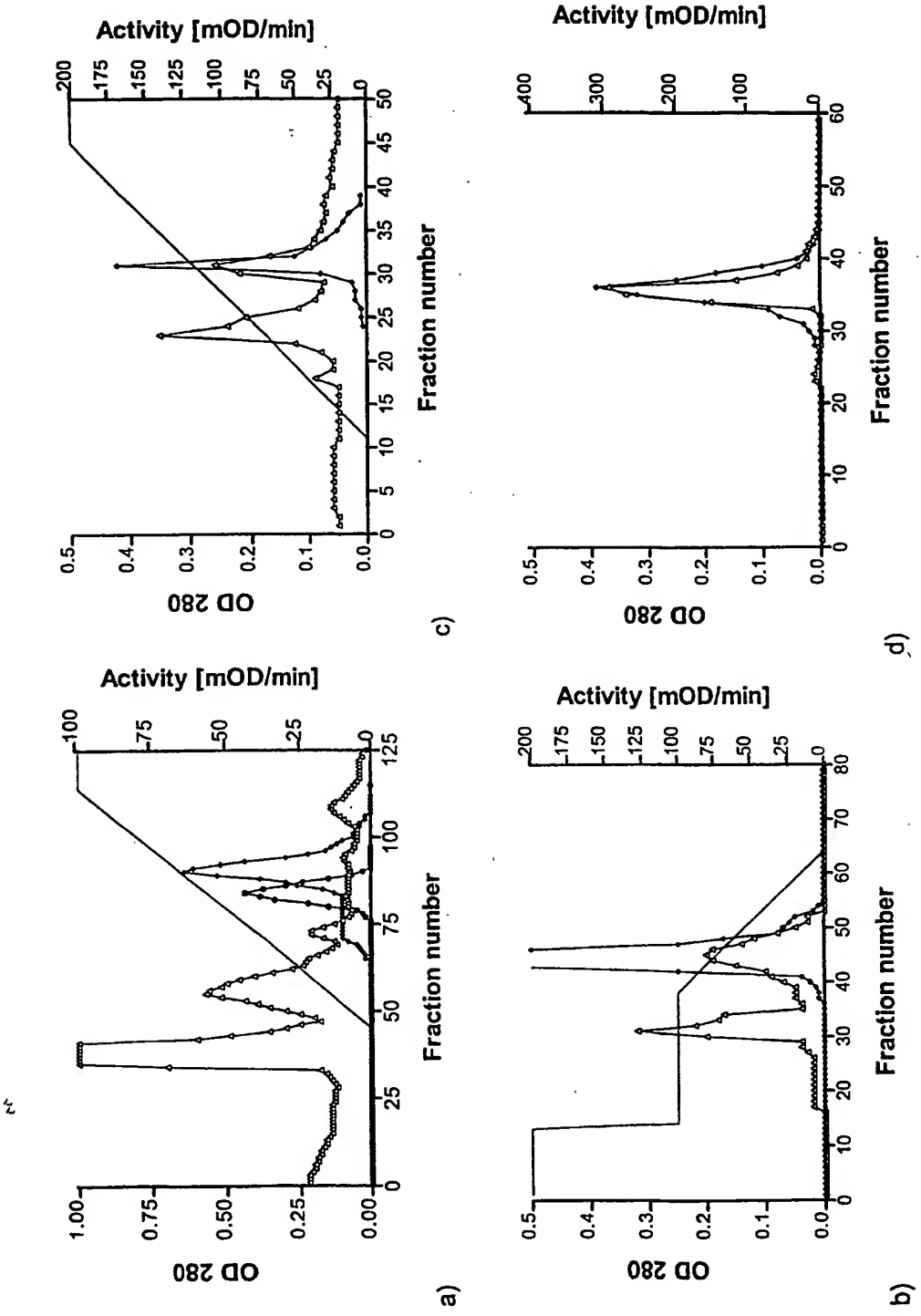


Fig. 2

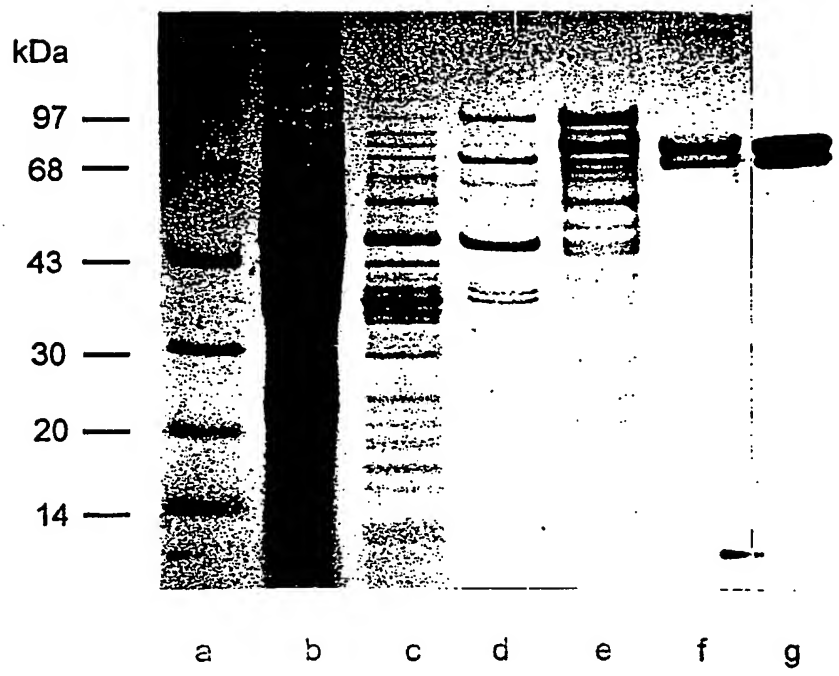
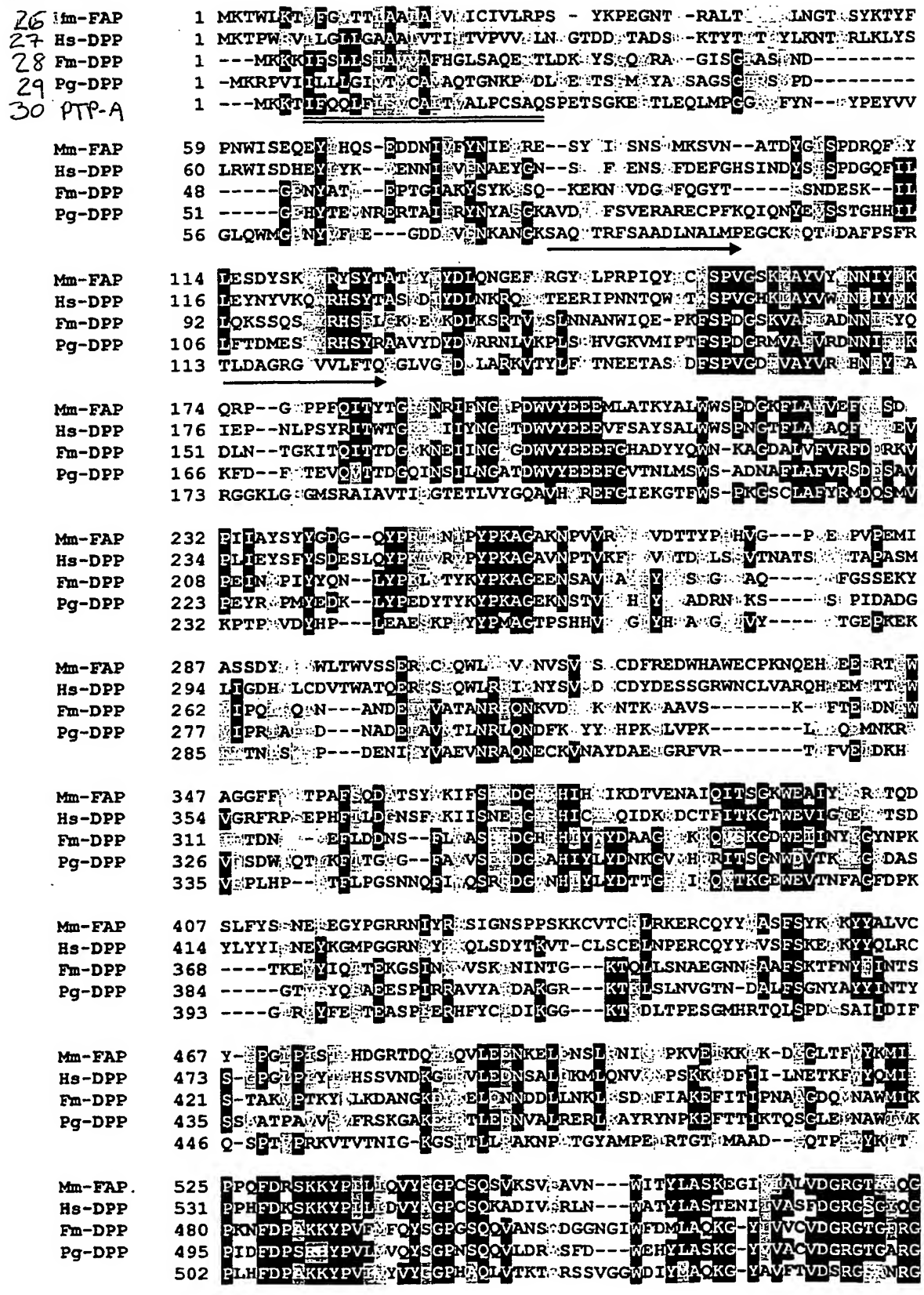


Fig. 3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29 Pg-DPP
30 PTP-A
Mm-FAP
Hs-DPP
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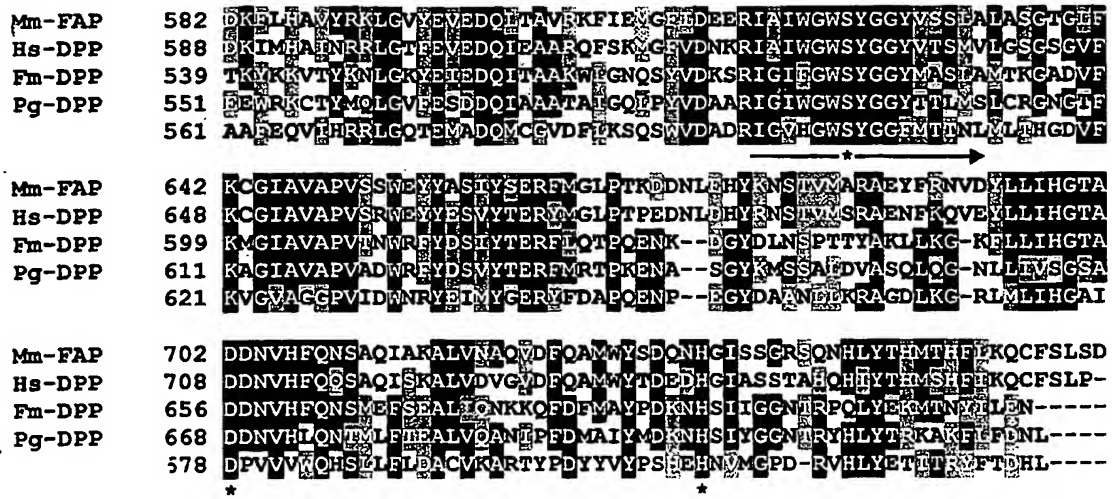
Pg-DPP

Fig. 3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621
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Hs-DPP 708
Fm-DPP 656
Pg-DPP 668
578

Fig. 4

```
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   DPP 499 VDAARIGIWGWSYGGYTT...NLLIVSGSADDNVHLQNTMLFTEALVQANIPFDMAIYMDKNHSIYGGNTR 661  32
DPP-H1 350 VDPDRIAIYGASHGGYAT...PLFVVQGANDPRVNINESDQIVTALRARGFEVPYMVKYNEGHGFHREENS 524  33
DPP-H2 640 VNGKKVGCFGASYGGFMT...PLLLLHGSVDTNVPTAESVNLYNALKILGREVEFIEFTEQDHFILEPERR 810  34
DPP-H3 495 VDGDRIGAVGASYGGFSV...PILMIHGELDFRIILASQAVAAFDAAQLRGVPSEMLIYPDENHWVLQPQNA 667  35
                       *                    *                          *
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Fig 5

Influence of Pefablock- serine proteinase inhibitor on *P. gingivalis* growth.

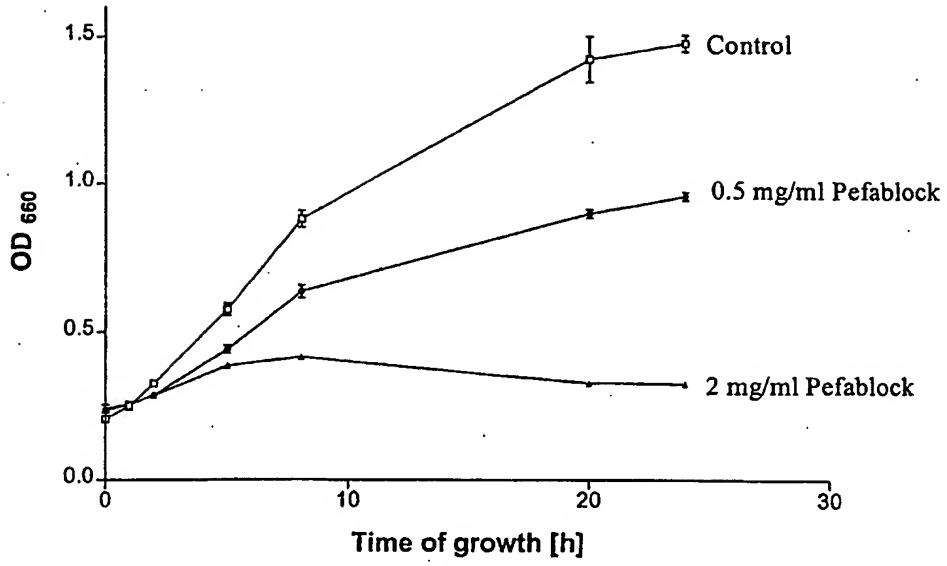

Fig. 6

SEQ ID NO:

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44 101PP    1 MKKSLLMLLLSAATISSIEAQTIQQMKAGGPWPVRAAFKTDTVGMNGSKYNPADLLRQAY
45 9PP      1 MNKKIFSMMAAS--IIGSAAMTPSAGTNTGEHLTPELEMTLSRVSEMALS--PDGKTAVY

126PP      55 VGLQWMGDNYVF-----IEGDDLVFNKANG------KSAQTTRFSAADLNALMPEGCKFQ
87PP       16 AILRY---NYAS-----GKAVDTLFSVER---------ARECPFKQIQ-N--------YE
65PP       14 SNIDG-----S------NTRDLTPFDGVK--------ASILNMLKEQK-D--------YM
101PP      61 DATDKDLRNVSADKDGRIACRKAGSKAERSEMAVYSFALTAEHFAKADIEVFGQGRMSLW
9PP        57 AVSFP--DVKT------NKATRELFTVNLD-------GSGRKQITDTESN----EYAPAW

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87PP       50 VSSTGHHILLFTDMES--IYRHSYRAAVYDYDVRRNLVKPL--SEHVGKVMIPTFSF---
65PP       46 IISMNK-----NNPQ----IFEPYKLNVVTEELTQLYEN----KDAANPIQGYEFDK---
101PP     121 LDDKQIGIADSPNSKCDTTIRFSASLSLVPGTHHLLLKSLLLEGDTTATDVRVVLKPKTA
9PP        98 MADGKR-TAFMSNEGG----SMQLWVMNADGTERRQLSN------IEGGITGFLESF---

126PP     157 -V---------G-DRVAYVRNHN-LYIARG--GKLGEGMSRAIAVTIDGTETLVYGQA--
87PP      103 -D---------G-RMVAFVRDNN-IFIK-----KFDFDTE--VQVTTDGQINSILNGATD
65PP       90 -D---------G-ELRGYSK----------------------LVNGIESELYYKD--
101PP     181 RDSSALYPNYTSKERISLKMMSGTFLSGGSLSPTGKYVLTSYRVSRDNKPAVTYNQLRD
9PP       144 -D---------E-KQVLFTND----------------------IKFGKRTKDIYPDLDK

126PP     201 -VHQREFGIEKG--TFSSPKGSCLAFYKM----------DQS-MVKPTPIVDYH----PL
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65PP      153 DK-TRIVLYDLKQN---K---IIREIPANE---DY-DVSGLHLS-RK-------------
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9PP       247 -MAVSLS--TN---SDIYI--YNLASGRTHNISEGMVGYDTYPK----------FSPD

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65PP      224 GKE--FSVV----E-------------------YDD----------------------
101PP     421 GKH--LIVMGS-ALASGNIGLNLKSGVTPNSYLKQFFLFDLSTRKATALTKNFNPSVSAG
9PP       287 GKS--IAWISMERDGYES-------------DLKRLFVADLAIGKRTHVNPTFDYNVDMI

126PP     389 -FDPKGTRLYYESTEASPLERHFYCIDINGGKTKDLTP-ESGMHRTQLSPD-GSAIIDIF
87PP      333 -VDASGT-VFYQSAESPIRRAVYAIDAKGRKTK-LSL-NVGTNDALFSGN-YAYYINTY
65PP      235 --FE--T-HLLIAVDSDKLYGTYYQFFTR---TK----------KFT-----LLYD-L
101PP     478 RFDRKNN-YYYFRAENG-SRKQLYRLELKTLEISQLQTGEDVVQWFGVAADNGAVWYSGQ
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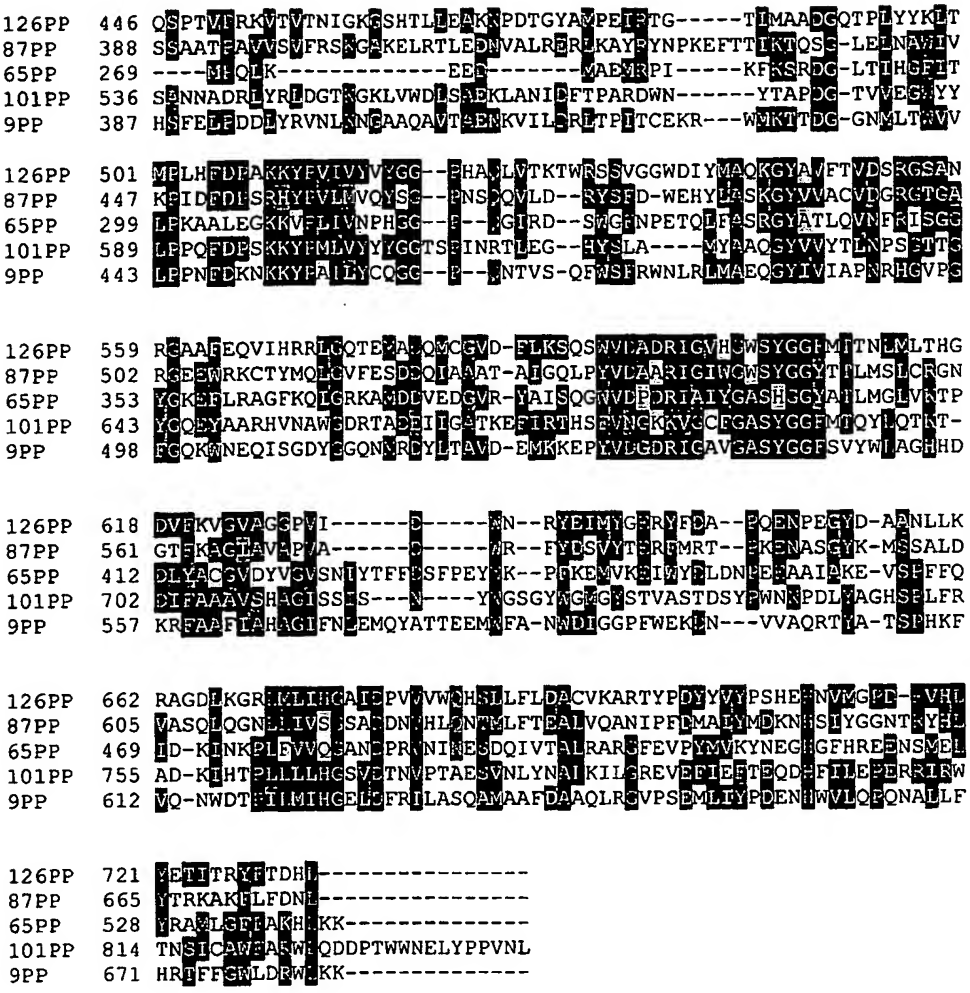
Fig. 6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126PP 662
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Fig 7

P. gingivalis W 83 PTP sequence

SEQ ID NO: 38
SEQ ID NO: 30

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13363 ttttataacttttaccccgaatacgtggtcggtttgcaatggatg
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Fig. 7

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Fig 7

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SQ ID NO:38

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AATGTGATGGGGCCGGACAGAGTACATTTGTATGAAACAATAACCCGTTATTTCACAGATCACTTATGA
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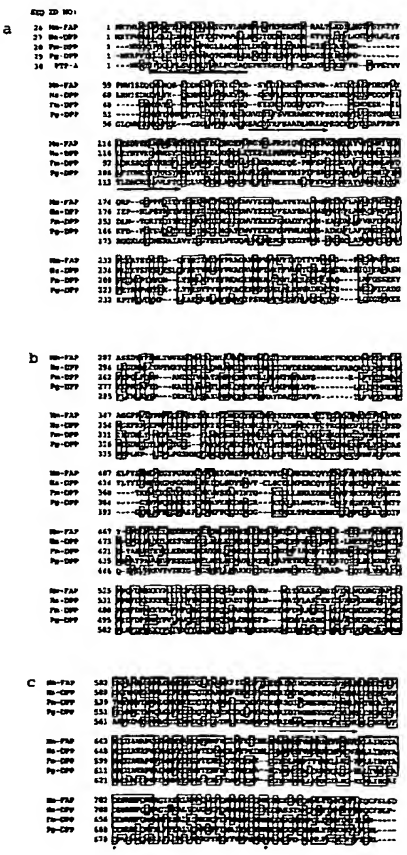
(54) Title: BACTERIAL PROLYL PEPTIDASES AND METHODS OF USE



(57) Abstract: The present invention provides isolated polypeptides, prolyl tripeptidyl-peptidases, and active analogs, active fragments or active modifications thereof, having amidolytic activity for cleavage of a peptide bond present in a target peptide having at least 30 amino acids. Isolated nucleic acid fragments encoding isolated prolyl tripeptidyl-peptidases are also provided, as are methods of reducing growth of a bacterium by inhibiting a prolyl tripeptidyl-peptidase.

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BACTERIAL PROLYL PEPTIDASES AND METHODS OF USE

CONTINUING APPLICATION DATA

This patent application claims the benefit of U.S. provisional patent application No. 60/123,148, filed March 5, 1999, which is incorporated by reference herein.

GOVERNMENT FUNDING

The present invention was made with government support under Grant No. DE 09761, awarded by the National Institutes of Health. The Government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

Porphyromonas gingivalis (formerly *Bacteroides gingivalis*) is an obligately anaerobic bacterium which is implicated in periodontal disease. *P. gingivalis* produces several distinct proteolytic enzymes, many of which are recognized as important virulence factors. A number of physiologically significant proteins, including collagen, fibronectin, immunoglobulins, complement factors C3, C4, C5, and B, lysozyme, iron-binding proteins, plasma proteinase inhibitors, fibrin and fibrinogen, and factors of the plasma coagulation cascade system, are hydrolyzed by *P. gingivalis* proteases. Broad proteolytic activity plays a role in the evasion of host defense mechanisms and the destruction of gingival connective tissue in progressive periodontitis.

Progressive periodontitis is characterized by acute tissue degradation promoted by collagen digestion and a vigorous inflammatory response characterized by excessive neutrophil infiltration. Gingival crevicular fluid accumulates in periodontitis as periodontal tissue erosion progresses at the foci of the infection, and numerous plasma proteins are exposed to proteinases expressed by the bacteria at the injury site. Neutrophils are recruited to the gingiva, in part, by the humoral chemotactic factor C5a. The complement components C3 and C5 are activated by complex plasma proteases with "trypsin-like" specificities called convertases. The human plasma convertases

cleave the α-chains of C3 and C5 at a specific site generating biologically active factors known as anaphylatoxins (i.e. C3a and C5a). The anaphylatoxins are potent proinflammatory factors exhibiting chemotactic and/or spasmogenic activities as well as promoting increased vascular permeability. The larger products from C3 and C5 cleavage (i.e. C3b and C5b) participate in functions including complement cascade activation, opsonization, and lytic complex formation.

Recent studies have indicated that this periodontopathogen produces at least seven different enzymes belonging to the cysteine and serine catalytic classes of peptidases, among which three cysteine proteinases (gingipains) are predominant (Potempa, J., et al. (1995) *Prospect. Drug Discovery and Design* 2, 445-458). The gingipains are the best characterized group of *P. gingivalis* enzymes as their structure, function, enzymatic properties and pathological significance are known. From *in vitro* studies it is apparent that two gingipains R (also referred to generally as "Arg-gingipains" and more specifically as RgpA and RgpB), enzymes specific for cleavage at Arg-Xaa peptide bonds, have a significant potential to contribute to the development and/or maintenance of a pathological inflammatory state in infected periodontal pockets through: (*i*) activation of the kallikrein-kinin cascade, (*ii*) the release of neutrophil chemotactic activity from native and oxidized C5 of the complement pathway, and (*iii*) activation of factor X. In addition, gingipain K (also referred to as "Lys-gingipain"), an enzyme which cleaves Lys-Xaa peptide bonds, degrades fibrinogen. This may add to a bleeding on probing tendency associated with periodontitis. Finally, the presence of a hemagglutinin/adhesion domain in the non covalent multiprotein complexes of RgpA and gingipain K suggests participation of these enzymes in the binding of *P. gingivalis* to extracellular matrix proteins which may facilitate tissue invasion by this pathogen.

In comparison to the gingipains, relatively little is known about other cysteine proteinases produced by *P. gingivalis*. Two genes, referred to as *tpr* and *prtT* have been cloned and sequenced and although they encode a putative papain-like and streptopain-like cysteine proteinases, respectively, neither has been purified and characterized.

The presence of serine proteinase activity in cultures of *P. gingivalis* has been known for several years; however, only limited information is available about such enzymes. Indeed, a serine endopeptidase has been isolated from culture media, although it was only superficially characterized (Hinode D., et al., (1993) *Infect. Immun.* 59, 3060-3068). On the other hand, an enzyme referred to as glycylprolyl peptidase (DPP IV) was found to be associated with bacterial surfaces and two molecular mass forms of this peptidase have been described. This enzyme has also been shown to possess the ability to hydrolyze partially degraded type I collagen, releasing the Gly-Pro dipeptide, and it was suggested that, in collaboration with collagenase, glycylprolyl peptidase may contribute to the destruction of the periodontal ligament (Abiko, Y., et al. (1985) *J.Dent. Res.* 64, 106-111). In addition to this potential pathological function, glycylprolyl peptidase may also play a vital role in providing *P. gingivalis* with dipeptides which can be transported inside the cell and serve as a source of carbon, nitrogen, and energy for this asaccharolytic organism. Recently, a gene encoding glycylprolyl peptidase in *P. gingivalis* has been cloned and sequenced, and it is now apparent that this enzyme is homologous to dipeptidyl-peptidase IV (DPP-IV) from other organisms (Kiyama, M., et al. (1998) 1396, 39-46). The nucleotide sequence of the genome of this bacterium is currently being determined by The Institute for Genomic Research, and is available at www.tigr.org.

SUMMARY OF THE INVENTION

The present invention is directed to an isolated prolyl tripeptidyl-peptidase, active analog, active fragment, or active modification thereof having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids. Alternatively, the isolated prolyl tripeptidyl-peptidase, active analog, active fragment, or active modification thereof is isolated from *P. gingivalis*. Typically, amidolytic activity is determined with a prolyl tripeptidyl-peptidase:target polypeptide ratio of at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours. The peptide cleaved by the isolated prolyl tripeptidyl-peptidase can include the sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID

NO:7, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:11, H-Ala-Arg-Pro-Ala-D-Lys-amide, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:25, or SEQ ID NO:37. The amino acid sequence of the isolated prolyl tripeptidyl-peptidase can include the amino acid sequence GXSXXG (SEQ ID NO:39), the amino acid sequence GXSXGG (SEQ ID NO:40), or the amino acid sequence of SEQ ID NO:30.

Another aspect of the invention is an isolated polypeptide, active analog, active fragment, or active modification thereof having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids. Typically, the polypeptide:target polypeptide ratio of at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours.

The invention is also directed to an isolated polypeptide comprising an amino acid sequence having a percentage amino acid identity of greater than 35 % with SEQ ID NO:30.

An alternative aspect of the invention is an isolated nucleic acid fragment encoding a prolyl-tripeptidyl peptidase, active analog, active fragment, or active modification thereof, having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids. Typically, the prolyl tripeptidyl-peptidase:target polypeptide ratio of at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours. The nucleic acid fragment can have a nucleotide sequence comprising SEQ ID NO:38. A complement of the nucleic acid fragment can hybridize to SEQ ID NO:38 under hybridization conditions of 0.5 M phosphate buffer, pH 7.2, 7 % SDS, 10 mM EDTA, at 68°C, followed by three for 20 minutes washes in 2x SSC, and 0.1 % SDS, at 65°C, wherein at least about 20 nucleotides of the complement hybridize.

Another aspect of the invention is an isolated nucleic acid fragment encoding a polypeptide that includes an amino acid sequence having a percentage amino acid identity of greater than 35 % with SEQ ID NO:30.

The invention is also directed at a method of identifying an inhibitor of a prolyl-tripeptidyl peptidase, active analog, active fragment, or active modification thereof, including identifying a molecule that inhibits the

amidolytic activity of the prolyl-tripeptidyl peptidase. The inhibitor is identified by incubating the prolyl-tripeptidyl peptidase with the molecule under conditions that promote amidolytic activity of the prolyl-tripeptidyl peptidase and determining if the amidolytic activity of the prolyl-tripeptidyl peptidase is inhibited relative to the amidolytic activity in the absence of molecule.

An aspect of the invention is a method of reducing growth of a bacterium. This method includes inhibiting a prolyl tripeptidyl-peptidase, active analog, active fragment, or active modification thereof, or a prolyl dipeptidyl-peptidase, active analog, active fragment, or active modification thereof. The method includes contacting the prolyl tripeptidyl-peptidase with an inhibitor of the prolyl tripeptidyl-peptidase. The method can be used to protect an animal from a periodontal disease caused by *P. gingivalis* including administering to the animal the inhibitor. The disease can be selected from the group consisting of gingivitis and periodontitis. The inhibitor can be administered by a method selected from the group consisting of subgingival application and controlled release delivery.

Another aspect of the invention is an immunogenic composition including an isolated prolyl tripeptidyl-peptidase, or an antigenic analog, antigenic fragment, or antigenic modification thereof, the prolyl tripeptidyl-peptidase having amidolytic activity for cleavage of a peptide bond present in a target peptide having at least 4 amino acids. Typically, the prolyl tripeptidyl-peptidase:target polypeptide ratio is at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours. The immunogenic composition can include an adjuvant.

The invention is also directed to a composition including an inhibitor of an isolated prolyl tripeptidyl-peptidase and a pharmaceutically acceptable carrier.

Additional aspects of the invention include a dipeptidyl peptidase having an amino acid sequence including SEQ ID NO:43, SEQ ID NO:44, or SEQ ID NO:45.

Definitions

"Polypeptide" as used herein refers to a polymer of amino acids and does not refer to a specific length of a polymer of amino acids. Thus, for example, the terms peptide, oligopeptide, protein, and enzyme are included within the definition of polypeptide. This term also includes post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations, and the like. A polypeptide can be produced by an organism, or produced using recombinant techniques, or chemically or enzymatically synthesized.

"Peptidase," "proteinase," and "protease" all refer to enzymes that catalyze the hydrolysis of peptide bonds in a polypeptide. A "peptide bond" or "amide bond" is a covalent bond between the alpha-amino group of one amino acid and the alpha-carboxyl group of another amino acid. "Peptidase inhibitor," "proteinase inhibitor," "protease inhibitor," and "inhibitor" all refer to molecules that inhibit a peptidase that catalyzes the hydrolysis of peptide bonds in a polypeptide.

As used herein, the term "isolated" means that a polypeptide or a nucleic acid fragment has been either removed from its natural environment, produced using recombinant techniques, or chemically or enzymatically synthesized. Preferably, the polypeptide or nucleic acid fragment is purified, i.e., essentially free from any other polypeptides or nucleic acid fragments and associated cellular products or other impurities.

"Amidolytic activity" refers to the ability of a polypeptide to catalyze the hydrolysis of at least one peptide bond in a polypeptide. The term "cleavage" can also be used to refer to the hydrolysis of a peptide bond in a polypeptide. "Prolyl-tripeptidyl peptidase" and "PTP" refer to a polypeptide having a particular "amidolytic activity". A "prolyl-tripeptidyl peptidase" is able to hydrolyze the peptide bond between the proline and the Yaa residues in a target polypeptide with the general formula NH_2-Xaa-Xaa-Pro-Yaa-$(Xaa)_n$ (SEQ ID NO:25), wherein Xaa is a natural or modified amino acid, Yaa is a natural or modified amino acid except proline, and the α-amino of the amino terminal residue is not blocked. A "prolyl tripeptidyl-peptidase" does not have to cleave all members of the target peptide. The term "natural amino acid" refers to the 20 amino acids typically produced by a cell. The term "modified amino acid"

refers to, for instance, acetylation, hydroxylation, methylation, amidation, and the attachment of carbohydrate or lipid moieties, cofactors, and the like.

A "target polypeptide" is a polypeptide that is the potential substrate of the amidolytic activity of a prolyl tripeptidyl-peptidase.

An active analog, active fragment, or active modification of a polypeptide of the invention is one that has amidolytic activity by hydrolysis of a peptide bond present in the target polypeptide as described herein. Active analogs, fragments, and modifications are described in greater detail herein.

"Nucleic acid fragment" as used herein refers to a linear polymeric form of nucleotides of any length, either ribonucleotides or deoxynucleotides, and includes both double- and single-stranded DNA and RNA. A nucleic acid fragment may include both coding and non-coding regions that can be obtained directly from a natural source (e.g., a microorganism), or can be prepared with the aid of recombinant or synthetic techniques. A nucleic acid molecule may be equivalent to this nucleic acid fragment or it can include this fragment in addition to one or more other nucleotides or polynucleotides, For example, the nucleic acid molecule of the invention can be a vector, such as an expression of cloning vector.

"Percentage amino acid identity" refers to a comparison of the amino acids of two polypeptides as described herein.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1. Purification of the prolyl tripeptidyl peptidase from the acetone precipitate of the *P. gingivalis* cell extracts. Absorbance at 280 nm (open triangles), amidolytic activity against H-Ala-Phe-Pro-pNA (closed diamonds), and H-Gly-Pro-pNA (closed circles). *(a)* Separation of PTP-A on hydroxyapatite. *(b)* Separation of PTP-A on Phenyl-Sepharose HP. *(c)* Separation of PTP-A on MonoQ FPLC. *(d)* Chromatofocusing of PTP-A on Mono-P.

Fig. 2. SDS -PAGE of fractions from purification of PTP-A and the autoradiography of the purified enzyme. *Lane a*, molecular mass markers (phosphorylase B, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa; α-lactalbumin, 14

kDa); *lane b*, acetone precipitate from Triton X-100 extract of *P. gingivalis*, *lane c*: hydroxyapatite column eluate; *lane d*, Phenyl-Sepharose column eluate; *lane e*, MonoQ column eluate; *lane f*, purified PTP-A from MonoP column wash; *lane g*, autoradiograph of ^{3}H-DFP labeled enzyme exposed for 96 h to X-ray film. All samples were reduced and boiled prior to PAGE analysis.

Fig. 3. Multiple sequence alignment of *P. gingivalis* PTP-A (PTP-A) and its bacterial and eukaryotic homologues. Pg-DPP, DPP from *P. gingivalis* (Kiyama, M., et al., (1998) *Bioch. Bioph. Acta* 1396, 39-46) containing an amino-terminal sequence corrected according to the *P. gingivalis* W83 genome data available from The Institute of Genomic Research at www.tigr.org); Fm-DPP, DPP from *Flavobacterium meningosepticum*; Hs-DPP, human DPP IV; and Mm-FAP, mouse fibroblast activation protein. Peptide sequences obtained from PTP-A analysis described herein are indicated with arrows (note that the sequence of the peptide 81-97 corresponds to the N-terminus of the lower molecular weight form of PTP-A); catalytic triad is marked with asterisks; and the proposed PTP-A membrane-anchoring N–terminal α-helix is double-underlined. Homologous regions (i.e., regions of identical amino acids and/or conservative substitutions) are highlighted. Identical regions are shown as white letters on a black background.

Fig. 4. Comparison of *P. gingivalis* PTP-A and DPP active site domains to corresponding sequences of three putative homologues identified within the *P. gingivalis* genome (DPP-H1, DPP-H2 and DPP-H3). Sequences of *P. gingivalis* PTP-A, DPP, DPP-H1, DPP-H2, and DPP-H3 were obtained from conceptual translation of the following open reading frames retrieved from The Institute for Genomic Research (TIGR) unfinished P. gingivalis genome database: gnl | TIGR | *P. gingivalis* contig 126 (positions 13 228 – 15 426), contig 87 (positions 6 424 – 4 399), contig 65 (positions 161 – 1 786), contig 101 (positions 8 895 – 6 845), and contig 9 (positions 4 216 – 2 162), respectively. Residues predicted as catalytic triads are marked with asterisks. Homologous regions (i.e., regions of identical amino acids and/or conservative substitutions) are highlighted.

Identical regions are shown as white letters on a black background. Similar regions (i.e., conservative substitutions) are shown as white letters on a grey background.

Fig. 5. Influence of Pefabloc-serine proteinase inhibitor on *P. gingivalis* growth.

Fig. 6. Comparison of *P. gingivalis* PTP-A and DPP to sequences of three putative homologues identified within the *P. gingivalis* genome (DPP-H1, DPP-H2 and DPP-H3). Sequences of *P. gingivalis* PTP-A (126PP), DPP (87PP), DPP-H1 (65PP), DPP-H2 (101PP), and DPP-H3 (9PP) were obtained as described in Fig. 4. Homologous regions (i.e., regions of identical amino acids and/or conservative substitutions) are highlighted. Identical regions are shown as white letters on a black background. Similar regions (i.e., conservative substitutions) are shown as white letters on a grey background.

Fig. 7. Nucleotide sequence (SEQ ID NO:38) and amino acid sequence (SEQ ID NO:30) of PTP-A.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides isolated polypeptides, preferably isolated prolyl peptidases, more preferably prolyl dipeptidyl-peptidases and prolyl tripeptidyl-peptidases, most preferably prolyl-tripeptidyl peptidases, that have amidolytic activity by hydrolysis of a peptide bond present in a target polypeptide, where the bond is between a proline and an amino acid residue attached to the alpha-carboxyl group end of the proline.

When the prolyl peptidase is a prolyl tripeptidyl-peptidase, the peptidase has amidolytic activity by hydrolysis of a peptide bond present in a target polypeptide of the general formula NH_2-Xaa-Xaa-Pro-Yaa-$(Xaa)_n$ (SEQ ID NO:25), wherein Xaa is a natural or modified amino acid, Yaa is a natural or modified amino acid except proline, and the α-amino of the amino terminal residue is not blocked, wherein the peptide bond of the target polypeptide that is hydrolyzed is the Pro-Yaa peptide bond. Preferably, isolated polypeptides do not cleave a target peptide having a blocked α-amino of the amino terminal residue.

Preferably, the only peptide bond of the target peptide that is hydrolyzed is the Pro-Yaa bond. In increasing order of preference, isolated polypeptides can cleave a target peptide that is at least 4 amino acids or at least 300 Da, at least 10 amino acids or at least 750 Da, at least 20 amino acids or at least 1,500 Da, or at least 30 amino acids or at least 3,000 Da. Preferably, the prolyl-tripeptidyl peptidases cleave peptides 1, 2, 7, 8, and 10-15 shown in Table 3, human cystatin C, and interleukin 6.

When the prolyl peptidase is a prolyl dipeptidyl-peptidase, the peptidase has amidolytic activity by hydrolysis of a peptide bond present in a target polypeptide of the general formula NH_2-Xaa-Zaa-Yaa-$(Xaa)_n$ (SEQ ID NO:12), wherein Xaa is a natural or modified amino acid, Zaa is a proline or alanine, Yaa is a natural or modified amino acid except proline or hydroxyproline, and the α-amino of the amino terminal residue is not blocked, wherein the peptide bond of the target polypeptide that is hydrolyzed is the Zaa-Yaa peptide bond. Preferably, isolated polypeptides does not cleave a target peptide having a blocked α-amino of the amino terminal residue. Preferably, the only peptide bond of the target peptide that is hydrolyzed is the Pro-Yaa bond.

Due to their cyclic aliphatic character proline residues bestow unique conformational constraints on polypeptide chain structures, significantly affecting the susceptibility of proximal peptide bonds to proteolytic cleavage. Those proline residues, which often appear near the amino-termini of many biologically active peptides, may protect them against proteolytic degradation by peptidases with general specificity. A specialized group of proteolytic enzymes, typically referred to as prolyl peptidases, has evolved to cleave (i.e., hydrolyze) a peptide bond adjacent to a proline residue in a polypeptide. The peptide bond adjacent to a proline residue can be referred to as a prolyl-X bond, where prolyl is the proline residue, and X is an amino acid residue attached to the alpha-carboxyl group end of the proline. The bacterial prolyl peptidases can cleave a polypeptide to liberate a tripeptide or a dipeptide. Prolyl peptidases that do not cleave a target peptide if the α-amino of the amino terminal residue is blocked can be referred to as exopeptidases. The *in vivo* activity of these specialized proteolytic enzymes may have important physiological significance, because it may lead to inactivation of many biologically active peptides and/or transformation of the activity of other

biologically active peptides. In addition, hydrolysis of prolyl-X bonds in conjunction with general catabolic pathways should allow the complete re-utilization of amino acids by living organisms, including bacteria. However, prolyl peptidases from bacterial pathogens, if released into the host environment, may interfere with the physiological functions of biologically active polypeptides and, therefore, contribute to the pathogenicity of infectious disease.

The external (i.e., cell surface) localization and uncontrolled activity of bacterial peptidases, including prolyl peptidases, likely contributes significantly to run-away inflammation in the human host and the pathological degradation of connective tissue during periodontitis. For instance, working in concert bacterial prolyl peptidases (e.g., prolyl tripeptidyl peptidases and DPP IV) have the ability to completely degrade collagen fragments locally generated by endogenous or bacterial collagenases. Because type I collagen is the major component of periodontal ligament, its enhanced degradation by bacterial prolyl peptidases may contribute to loss of tooth attachment and periodontal pocket formation. Thus, there is a need in the art to characterize bacterial peptidases to facilitate the development of therapies to inhibit the activity of the bacterial peptidases.

The polypeptides of the present invention, preferably prolyl peptidases, can be used as a source of antibodies for inhibiting the peptidase activity and thereby possibly reducing periodontitis, loss of tooth attachment and periodontal pocket formation. Antibodies to prolyl peptidases can also be used to identify and/or isolate additional prolyl peptidases. Knowledge of prolyl peptidases can also be used to make inhibitors of prolyl peptidases and to make immunogenic compositions that could be used to elicit the production of antibodies to prolyl peptidases and thereby possibly reduce gingivitis, periodontitis, loss of tooth attachment, and/or periodontal pocket formation.

An example of a prolyl-tripeptidyl peptidase is prolyl-tripeptidyl peptidase A (SEQ ID NO:30) (also referred to as PTP-A) from *P. gingivalis*. Purified PTP-A has apparent molecular masses of 81.8 and 75.8 kDa. The lower molecular mass peptidase may be due to the proteolytic cleavage of the peptidase from the surface of *P. gingivalis*. PTP-A is a new member of clan SC, family S9 of serine peptidases. Clans of serine peptidases are grouped on the basis of the order of certain amino acids in the polypeptide that make up the "catalytic triad" which

plays a pivotal role peptidase activity. The members of the clan SC are characterized by the catalytic triad in the polypeptide in the order of serine, aspartic acid, and histidine. Members of the clan SC are also characterized by a tertiary structure including β/α/β units, and an α/β hydrolase fold. In addition to the catalytic triad order, the amino acid sequence GXSXXG (SEQ ID NO:39), where X is any amino acid and S is the active site serine, is a signature of all members of the clan SC with some distinguishing features specific for each family. Family S9 has the consensus sequence GXSXGG (SEQ ID NO:40). Besides this consensus sequence, there is a general similarity of primary structures which classifies peptidases to this family. For instance, peptidases of this family generally have two domains, an amino-terminal domain that contains a membrane binding domain, and a carboxy-terminal domain, also referred to as the catalytic domain. The catalytic domain contains the residues of the catalytic triad. Some members of the S9 family have only the catalytic domain.

The S9 family is diverged and divided in three subfamilies: S9A, cytosolic oligopeptidases from archae and eukaryotes; S9B, eukaryotic acylaminoacylpeptidases; and S9C, dipeptidyl peptidease IV from bacteria and eukaryotes. The catalytic domain of peptidases from family S9 typically begin at about residue 400 of SEQ ID NO:30 and include the remaining carboxy-terminal amino acids (see, e.g., Fulop, et al., (1998) *Cell* 94, 161-170). Despite structural similarities to peptidases from the S9 family, the tripeptidyl-peptidase activity of PTP-A is unusual for this family of enzymes, and no other known similar activity has so far been attributed to any other member of the S9 family. In fact, all strict tripeptidyl-peptidases belong only to the subtilisin family (S8) and S33 family of serine peptidases; however, they neither share a structural relationship with PTP-A nor have activity limited to cleavage after proline residues. In particular, there are no other known prolyl tripeptidyl peptidases with an activity that is increased by iodoacetamide relative to the same prolyl tripeptidyl peptidase in the absence of iodoacetamide under the same conditions. Iodoacetamide is a compound that is traditionally a peptidase inhibitor. Typically, the activity of a prolyl tripeptidyl peptidase is increased about two-fold. Furthermore, unlike oligopepidases, the prolyl tripeptidyl-peptidases of the present invention can cleave target peptidases having as few as 4 amino acids but also target peptides having at least 30 amino

acids or a molecular weight of at least 3,000 Da. In these respects, the *P. gingivalis* tripeptidyl peptidase is a unique enzyme, and the isolation and characterization of this novel bacterial prolyl peptidase will facilitate the development of therapies to inhibit the activity of the bacterial peptidases.

Examples of putative prolyl-dipeptidyl peptidases are DPP-H1 (SEQ ID NO:43), DPP-H2 (SEQ ID NO:44), and DPP-H3 (SEQ ID NO:45). These peptidases have a significant percentage amino acid similarity with DPP IV and PTP-A (see Fig. 6). Each dipeptidyl peptidase is expected to have enzymatic activity, as each has a well preserved catalytic triad (Fig. 4). DPP IV has been characterized and the gene encoding the peptidase has been cloned, however the substrate specificity has not been well characterized. DPP IV has been found to cleave SEQ ID NOs:6, 20, 23, and 24. DPP IV has been purified in two forms. One of the forms is a full length gene translation product containing a blocked amino-terminal residue. The second form had the amino-terminal amino acid sequence HSYRAAVYDYDVRRNLVKPLSEHVG (SEQ ID NO:48), which corresponds to residues 116-140 of DPP IV (Kiyama, M., et al. (1998) 1396, 39-46), indicating that it was proteolytically truncated on the amino-terminus.

In *P. gingivalis*, PTP-A and DPP IV activity is cell surface associated. While not intending to be limiting, it is conceivable that the enzyme is membrane anchored through a putative signal sequence which is not cleaved but remains as a membrane spanning domain similar to other members of the prolyl oligopeptidase family. However, a significant portion of the purified PTP-A has a truncated N-terminus, apparently due to cleavage by Lys-specific peptidase and likely to be an artifact which has occurred during the purification procedure. Nevertheless, membrane bound PTP-A and DPP IV is proteolytically cleaved and shed during cultivation of the bacteria, as indicated by variable amount of soluble activities found in cell free culture media. The cell surface localization of PTP-A supports a putative physiological function in providing nutrients for growing bacterial cells. The inability of asaccharolytic *P. gingivalis* to utilize free amino acids makes the bacterium entirely dependant on an external peptide supply. In this regard, PTP-A and DPP IV activities are probably very important, if not indispensable, for bacterial growth, and inhibition of prolyl tripeptidyl-peptidases and dipeptidyl-peptidases may inhibit the *in vivo* growth of organisms, including *P. gingivalis*.

For instance, treatment of *P. gingivalis* cultures in lagphase (i.e., the period after inoculation of a culture and before the organism begins to divide) and early logarithmic growth with the inhibitors PEFABLOCK and 3,4-dichloroisocoumarin inhibits growth of *P. gingivalis*.

Preferably, a polypeptide of the invention, preferably a prolyl peptidase, contains the amino acid sequence GXSXXG (SEQ ID NO:39), most preferably, GXSXGG (SEQ ID NO:40), where G is glycine, X is any amino acid, and S is the active site serine. The active site serine can be identified by, for instance, labeling with diisopropylfluorophosphate as described herein. Preferably, the catalytic domain of the prolyl tripeptidyl-peptidases of the invention begins at about residue 400 of SEQ ID NO:30 and includes the remaining carboxy-terminal amino acids and the corresponding amino acids of SEQ ID NOs:43-45 (see Fig. 6), more preferably, at about residue 502 of SEQ ID NO:30 and includes the remaining carboxy-terminal amino acids and the corresponding amino acids of SEQ ID NOs:43-45 (see Fig. 6), most preferably, at about residue 556 of SEQ ID NO:30 and includes the remaining carboxy-terminal amino acids and the corresponding amino acids of SEQ ID NOs:43-45 (see Fig. 6).

The invention further includes a polypeptide, preferably a prolyl tripeptidyl-peptidase, that shares a significant level of primary structure with SEQ ID NO:30. The two amino acid sequences (i.e., the amino acid sequence of the polypeptide and the sequence SEQ ID NO:30) are aligned such that the residues that make up the catalytic triad, i.e., the serine, aspartic acid, and the histidine, are in register, then further aligned to maximize the number of amino acids that they have in common along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to place the residues of the catalytic triad in register and to maximize the number of shared amino acids, although the amino acids in each sequence must nonetheless remain in their proper order. The percentage amino acid identity is the higher of the following two numbers: (a) the number of amino acids that the two sequences have in common within the alignment, divided by the number of amino acids in SEQ ID NO:30, multiplied by 100; or (b) the number of amino acids that the two sequences have in common within the alignment, divided by the number of amino acids in the candidate polypeptide, multiplied by 100. Preferably, a prolyl tripeptidyl peptidase has

greater than 35 % identity, more preferably at least about 40 % identity, most preferably at least about 45 % identity with SEQ ID NO:30. Preferably, amino acids 154-732 of SEQ ID NO:30 are used, more preferably amino acids 400-732 of SEQ ID NO:30 are used. An isolated polypeptide comprising an amino acid sequence having a percentage amino acid identity of greater than 35 % with SEQ ID NO:30.

In general, the amidolytic activity of the polypeptides of the invention, preferably prolyl peptidases, can be measured by assay of the cleavage of a target polypeptide in the presence of prolyl peptidase and a buffer. Preferably, the lower ratio of prolyl tripeptidyl-peptidase to target polypeptide is at least about 1:1, more preferably at least about 1:100, even more preferably at least about 1:1,000, most preferably at least about 1:10,000. Preferably, the higher ratio of prolyl peptidase to target polypeptide is no greater than about 1:10,000,000, more preferably no greater than about 1:1,000,000 and most preferably no greater than about 1:100,000. Buffers in which a prolyl peptidase is active are suitable for the assay. Preferably, the buffer is about 200 mM HEPES (N-2-hydroxyethylpeperazine,N'-2-ethansulfonic acid), more preferably about 50 mM HEPES, most preferably about 20 mM HEPES. Preferably, the pH of the buffer is at least about pH 6.0 and no greater than pH 8.0, more preferably about pH 7.5. Preferably, the temperature of the assay is at about 37°C. The assay can be carried out for at least about 1 minute to no greater than 24 hours. Preferably, the amidolytic activity of the prolyl peptidases are measured at a prolyl peptidase:target polypeptide ratio of at least about 1:100 to no greater than 1:1,000,000 in about 200 mM HEPES, about pH 7.5 at about 37°C for at least about 3 hours. In general, the time of the assay can vary depending on the substrate and enzyme:substrate ratio. Typically, target peptides are stable under these conditions, and typically it is difficult to detect background levels of hydrolysis in the absence of a prolyl peptidase. Preferably, the assay is allowed to continue until at least 1 % of the target peptide is hydrolyzed.

Prolyl-tripeptidyl peptidases of the present invention preferably are inhibited by a compound chosen from the group consisting of PEFABLOCK (4-(2-aminoethyl)-benzenesulfonyl-fluoride hydrochloride), diisopropylfluorophosphate, and 3,4-dichloroisocoumarin, more preferably PEFABLOCK and diisopropylfluorophosphate, and most preferably diisopropylfluorophosphate. The

peptidases of the present invention are preferably not inhibited by a compound chosen from the group consisting of leupeptin, antipain, E-64, pepstatin, α_1-proteinase inhibitor, α_1–antichymotrypsin and α_2–macroglobulin, most preferably. Significantly and unexpectedly, the amidolytic activity of a prolyl-tripeptidyl peptidase of the present invention is increased by iodoacetamide relative to the prolyl-tripeptidyl peptidase in the absence of iodoacetamide under the same conditions. Preferably, the effect of iodoacetamide on amidolytic activity is measured by incubating in 200 mM HEPES, pH 7.6, at least about 0.1 nM of the prolyl tripeptidyl-peptidase with the inhibitor for about 15 minutes, adding about 1 mM of H-Ala-Phe-Pro-pHA, and incubating for at least about 1 minute before assaying for amidolytic activity. Typically, at least about 1 mM to no greater than 100 mM of inhibitor is used.

The polypeptides of the invention include a polypeptide having SEQ ID NO:30, or an active analog, active fragment, or active modification of SEQ ID NO:30. An active analog, active fragment, or active modification of a polypeptide having SEQ ID NO:30 is one that has amidolytic activity by hydrolysis of the Pro-Yaa peptide bond present in a target polypeptide of the general formula NH_2-Xaa-Xaa-Pro-Yaa-$(Xaa)_n$ (SEQ ID NO:25). Active analogs of a polypeptide having SEQ ID NO:30 include prolyl-tripeptidyl peptidases having amino acid substitutions that do not eliminate hydrolysis of SEQ ID NO:25 at the Pro-Yaa peptide bond. Substitutes for an amino acid may be selected from other members of the class to which the amino acid belongs. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and tyrosine. Polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Examples of preferred conservative substitutions include Lys for Arg and *vice versa* to maintain a positive charge; Glu for Asp and *vice versa* to maintain a negative charge; Ser for Thr so that a free -OH is maintained; and Gln for Asn to maintain a free NH_2.

Active fragments of a prolyl-tripeptidyl peptidase of the invention include prolyl-tripeptidyl peptidases containing deletions or additions of one or more contiguous or noncontiguous amino acids such that the resulting polypeptide will

hydrolyze SEQ ID NO:25 at the Pro-Yaa peptide bond. An example of a fragment of a prolyl-tripeptidyl peptidase is a catalytic domain. Modified prolyl-tripeptidyl peptidases include prolyl-tripeptidyl peptidases that are chemically and enzymatically derivatized at one or more constituent amino acid, including side chain modifications, backbone modifications, and N- and C- terminal modifications including acetylation, hydroxylation, methylation, amidation, and the attachment of carbohydrate or lipid moieties, cofactors, and the like. Modified prolyl-tripeptidyl peptidases will hydrolyze SEQ ID NO:25 at the Pro-Yaa peptide bond.

Prolyl peptidases can be obtained by several methods. Isolation of a prolyl-tripeptidyl peptidase present on the surface of a cell producing the peptidase typically requires lysis of the cell followed by purification methods that are well known in the art. Alternatively, cels can be treated with a detergent, for instance Triton X-100, to remove the peptidase from the cell surface. The following are nonlimiting examples of suitable protein purification procedures: fractionation on immunoaffinity, ion-exchange, hydroxyapatite, Phenyl-Sepharose HP, MonoQ HR 5/5, or MonoP columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on an ion-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75. Preferably, isolation of a prolyl-tripeptidyl peptidase from *P. gingivalis* is accomplished using a combination of hydroxyapatite, Phenyl-Sepharose HP, MonoQ HR 5/5 and MonoP column chromatography steps as described herein.

Prolyl peptidases can also be isolated from organisms other than *P. gingivalis*. Other organisms can express a prolyl-tripeptidyl peptidase that is encoded by a coding region having similarity to the PTP-A coding region. A "coding region" is a linear form of nucleotides that encodes a polypeptide, usually via mRNA, when placed under the control of appropriate regulatory sequences. The boundaries of a coding region are generally determined by a translation start codon at its 5' end and a translation stop codon at its 3' end. "Regulatory region" refers to a nucleic acid fragment that regulates expression of a coding region to which a regulatory region is operably linked. Non limiting examples of regulatory regions include promoters, transcription initiation sites, translation start sites, translation

stop sites, and terminators. "Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A regulatory element is "operably linked" to a coding region when it is joined in such a way that expression of the coding region is achieved under conditions compatible with the regulatory region. Alternatively, other organisms can express a prolyl-tripeptidyl peptidase from a recombinant coding region encoding the peptidase. The identification of similar coding regions in other organisms can be accomplished as described herein. A prolyl-tripeptidyl peptidase can be isolated using purification methods that are well known in the art. Alternatively, the peptidase can be chemically synthesized using methods that are well known in the art including, for instance, solid phase synthesis. Examples of, for instance, coding and regulatory regions are described herein.

The expression of a prolyl-tripeptidyl peptidase by an organism other than *P. gingivalis* can be detected using specific substrates of the general formula NH_2-Xaa-Xaa-Pro-LG or NH_2-Xaa-Xaa-Pro-Yaa (SEQ ID NO:41), where LG is a leaving group. The leaving group can be a chromogenic or fluorogenic group known to the art. The expression of a prolyl-tripeptidyl peptidase by an organism and subsequent cleavage of a specific substrate results in a free amino acid or a free leaving group, each of which can be assayed using techniques known to those of skill in the art. Other methods can be based on immunogenic properties of PTP-A, for instance immunoassays and histochemistry, the detection of mRNA, and PCR related methods, all of which are known to one of skill in the art.

As described in the Examples, the amino acid sequence of the amino-terminal end of a PTP-A fragment was used to identify the nucleotide sequence of the PTP coding region. The nucleotide sequence was present in a publically available database containing the nucleotide sequence of the partially finished *P. gingivalis* W83 genome. However, even though the nucleotides that encode the *P. gingivalis* PTP-A were known, there was no indication that the nucleotides were in fact transcribed and translated. The data obtained from the database only contained the nucleotide sequence of a genomic clone; there was no disclosure that the nucleotides did or did not contain an open reading frame. Moreover, there is little data known to the art regarding regulatory regions required for either the transcription or the translation of a nucleotide sequence in *P. gingivalis*.

Thus, a person of ordinary skill, having the nucleotide sequence of the genomic clone, would not be able to predict that the open reading frame encoding PTP-A was transcribed or translated. Moreover, even if there was a suggestion that the open reading frame was both transcribed and translated, there is no suggestion that the polypeptide encoded by the open reading frame would have the novel activity of PTP-A.

Accordingly, the present invention is directed to a nucleic acid fragment encoding a polypeptide, particularly a prolyl-tripeptidyl peptidase, active analog, active fragment, or active modification thereof. The nucleic acid fragment can have a nucleotide sequence as shown in SEQ ID NO:38. Alternatively, nucleic acid fragments of the invention include those whose complement hybridize to SEQ ID NO:38 under standard hybridization conditions as described herein. During hybridization the entire nucleotide sequence of the complement can hybridize with SEQ ID NO:38. Preferably, at least about 20 nucleotides of the complement hybridize with SEQ ID NO:38, more preferably at least about 50 nucleotides, most preferably at least about 100 nucleotides.

Alternatively, the nucleic acid fragment can have a nucleotide sequence encoding a polypeptide having the amino acid sequence shown in SEQ ID NO:30. An example of the class of nucleotide sequences encoding such a polypeptide is SEQ ID NO:38. This class of nucleotide sequences is large but finite, and the nucleotide sequence of each member of the class can be readily determined by one skilled in the art by reference to the standard genetic code.

The identification of similar coding regions in other organisms can be accomplished by screening individual wild-type microorganisms for the presence of nucleotide sequences that are similar to the coding region of PTP-A, which is shown in SEQ ID NO:38. Screening methods include, for instance, hybridization of a detectably labeled probe with a nucleic acid fragment.

Standard hybridizing conditions are a modification of the conditions used by Church et al. ((1984) *Proc. Natl. Acad. Sci. USA* 81, 1991): 0.5 M phosphate buffer, pH 7.2, 7 % SDS, 10 mM EDTA, at 68°C, and three washes, each for 20 minutes in 2x SSC, 0.1 % SDS, at 65°C. Preferably, a probe will hybridize to the nucleotide sequence set forth in SEQ ID NO:38 under standard hybridizing conditions. Generally the probe does not have to be complementary to all the

nucleotides of the nucleic acid fragment as long as there is hybridization under the above-stated conditions.

"Complement" and "complementary" refer to the ability of two single stranded nucleic acid fragments to base pair with each other, where an adenine on one nucleic acid fragment will base pair to a thymine on a second nucleic acid fragment and a cytosine on one nucleic acid fragment will base pair to a guanine on a second nucleic acid fragment. Two nucleic acid fragments are complementary to each other when a nucleotide sequence in one nucleic acid fragment can base pair with a nucleotide sequence in a second nucleic acid fragment. For instance, 5'-ATGC and 5'-GCAT are complementary. The term complement and complementary also encompasses two nucleic acid fragments where one nucleic acid fragment contains at least one nucleotide that will not base pair to at least one nucleotide present on a second nucleic acid fragment. For instance the third nucleotide of each of the two nucleic acid fragments 5'-ATTGC and 5'-GCTAT will not base pair, but these two nucleic acid fragments are complementary as defined herein. Typically two nucleic acid fragments are complementary if they hybridize under the standard conditions referred to herein.

Preferred probes are nucleic acid fragments complementary to a coding region or another nucleotide sequence that encodes a prolyl-tripeptidyl peptidase. For instance, a probe can comprise a consecutive series of nucleotides complementary to a portion of SEQ ID NO:38. Preferably a probe is at least about 18 bases, more preferably at least about 21 bases, and most preferably at least about 24 bases in length. Particularly preferred probes are TTCGATCCGGCAAAGAAATATCCTGTTATTGTCTATGTTTACGGAGGAC CT (SEQ ID NO:36, GTGGATGCCGATAGAATAGGAGTACATGGCTGGAGCTATGGTGGCTTT (SEQ ID NO:37, and SEQ ID NO:38. Methods of detectably labeling a probe are well known to the art.

The nucleic acid fragment that is identified by the probe is further analyzed to determine if it encodes a polypeptide with amidolytic activity of the Pro-Yaa

peptide bond on a target polypeptide of the general formula NH_2-Xaa-Xaa-Pro-Yaa-$(Xaa)_n$ (SEQ ID NO:25). Another method for screening individual microorganisms for the presence of nucleotide sequences that are similar to the coding regions of the present invention is the polymerase chain reaction (PCR).

Individual wild-type microorganisms containing nucleic acid fragments encoding a prolyl-tripeptidyl peptidase can also be identified using antibody. Preferably the antibody is directed to PTP-A. The production of antibodies to a particular polypeptide is known to a person of skill in the art, and is further detailed herein.

The use of hybridization of a probe to a coding region present in individual wild-type microorganisms can be used as a method to identify a coding region identical or similar to a coding region present in SEQ ID NO:38. The coding region can then be isolated and ligated into a vector as described below. Two nucleic acid sequences are "similar" if the two nucleic acid sequences can be aligned so that the number of identical amino acids along the lengths of their sequences are optimized. Preferably, two nucleotide acid sequences have, in increasing order of preference, preferably at least about 90 %, at least about 92 %, at least about 94%, at least about 96%, most preferably at least about 98% identity.

As mentioned above, a nucleic acid fragment of the invention can be inserted in a vector. Construction of vectors containing a nucleic acid fragment of the invention employs standard ligation techniques known in the art. See, e.g., Sambrook et al, *Molecular Cloning: A Laboratory Manual.*, Cold Spring Harbor Laboratory Press (1989) or Ausubel, R.M., ed. *Current Protocols in Molecular Biology* (1994). A vector can provide for further cloning (amplification of the nucleic acid fragment), i.e., a cloning vector, or for expression of the polypeptide encoded by the coding region, i.e., an expression vector. The term vector includes, but is not limited to, plasmid vectors, viral vectors, cosmid vectors, or artificial chromosome vectors. Typically, a vector is capable of replication in a bacterial host, for instance *E. coli*. Preferably the vector is a plasmid.

Selection of a vector depends upon a variety of desired characteristics in the resulting construct, such as a selection marker, vector replication rate, and the like. Suitable plasmids for expression in *E. coli*, for example, include pUC(X), pKK223-3, pKK233-2, pTrc99A, and pET-(X) wherein (X) denotes a vector family in which

numerous constructs are available. pUC(X) vectors can be obtained from Pharmacia Biotech (Piscataway, NH) or Sigma Chemical Co. (St. Louis, MO). pKK223-3, pKK233-2 and pTrc99A can be obtained from Pharmacia Biotech. pET-(X) vectors can be obtained from Promega (Madison, WI) Stratagene (La Jolla, CA) and Novagen (Madison, WI). To facilitate replication inside a host cell, the vector preferably includes an origin of replication (known as an "ori") or replicon. For example, ColE1 and P15A replicons are commonly used in plasmids that are to be propagated in *E. coli*.

An expression vector optionally includes regulatory regions operably linked to the coding region. The invention is not limited by the use of any particular promoter, and a wide variety are known. Promoters act as regulatory signals that bind RNA polymerase in a cell to initiate transcription of a downstream (3' direction) coding region. The promoter used in the invention can be a constitutive or an inducible promoter. It can be, but need not be, heterologous with respect to the host cell. Preferred promoters for bacterial transformation include *lac*, *lac*UV5, *tac*, *trc*, T7, SP6 and *ara*.

An expression vector can optionally include a Shine Dalgarno site (e.g., a ribosome binding site), and a start site (e.g., the codon ATG) to initiate translation of the transcribed message to produce the enzyme. It can also include a termination sequence to end translation. A termination sequence is typically a codon for which there exists no corresponding aminoacetyl-tRNA, thus ending polypeptide synthesis. The nucleic acid fragment used to transform the host cell can optionally further include a transcription termination sequence. The rrnB terminators, which is a stretch of DNA that contains two terminators, T1 and T2, is an often used terminator that is incorporated into bacterial expression systems (J. Brosius et al., (1981) *J. Mol. Biol.* 148 107-127).

The nucleic acid fragment used to transform the host cell optionally includes one or more marker sequences, which typically encode a polypeptide that inactivates or otherwise detects or is detected by a compound in the growth medium. For example, the inclusion of a marker sequence can render the transformed cell resistant to an antibiotic, or it can confer compound-specific metabolism on the transformed cell. Examples of a marker sequence are sequences that confer resistance to kanamycin, ampicillin, chloramphenicol, and tetracycline.

Antibodies can be produced to a polypeptide having the sequence of SEQ ID NOs:30, 43, 44 or 45, or a polypeptide having a percentage amino acid identity as described herein. Alternatively, antibodies can be made to an antigenic analog, antigenic fragment, or antigenic modification of a polypeptide having the sequence of SEQ ID NOs:30, 43, 44 or 45. An antigenic analog, antigenic fragment, or antigenic modification of a polypeptide having SEQ ID NOs:30, 43, 44 or 45 is one that generates an immune response in an animal. Antigenic analogs of a polypeptide having SEQ ID NOs:30, 43, 44 or 45 include prolyl peptidases having amino acid substitutions that do not eliminate peptide antigenicity in an animal. Substitutes for an amino acid may be selected from other members of the class to which the amino acid belongs, as described herein. Fragments of a prolyl peptidase of the invention include prolyl peptidases containing deletions or additions of one or more contiguous or noncontiguous amino acids such that the resulting polypeptide will generate an immune response in an animal. An example of a fragments of a prolylpeptidase is a catalytic domain. Modified prolyl peptidases include prolyl peptidases that are chemically and enzymatically derivatized at one or more constituent amino acids, including side chain modifications, backbone modifications, and N- and C- terminal modifications including acetylation, hydroxylation, methylation, amidation, and the attachment of carbohydrate or lipid moieties, cofactors, and the like.

Accordingly, an aspect of the invention is an immunogenic composition comprising an isolated prolyl peptidase, or an antigenic analog, antigenic fragment, or antigenic modification thereof, preferably a prolyl tripeptidyl-peptidase. The prolyl tripeptidyl-peptidase preferably has amidolytic activity for cleavage of the Pro-Yaa peptide bond present in a target polypeptide with the general formula NH_2-Xaa-Xaa-Pro-Yaa-$(Xaa)_n$ (SEQ ID NO:25), wherein the amidolytic activity is measured at a prolyl tripeptidyl-peptidase:target polypeptide ratio of at least about 1:100 to no greater than about 1:1,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours.

The immunogenic composition can further include excipients or diluents that are pharmaceutically acceptable as carriers and compatible with the immunogenic composition. The term "pharmaceutically acceptable carrier" refers to a carrier(s) that is "acceptable" in the sense of being compatible with the other

ingredients of a composition and not deleterious to the recipient thereof. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the immunogenic composition may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the immune-stimulating composition.

The immunogenic composition can be used in a method for protecting an animal from a disease caused by *P. gingivalis*. This method includes administering the immunogenic composition and eliciting antibodies to a prolyl peptidase, antigenic analog, antigenic fragment, or antigenic modification. The diseases that can be treated in this manner include periodontal diseases, which includes gingivitis and periodontitis. Clinical hallmarks of periodontitis include lose of tooth attachment and periodontal pocket formation.

Alternatively and preferably, periodontal diseases can be treated by the use of inhibitors of a prolyl peptidase. An inhibitor of a prolyl peptidase, preferably a prolyl tripeptidyl-peptidase, can be present in a composition that preferably contains a pharmaceutically acceptable carrier. For instance, inhibitors can be applied systemically, subgingivally (e.g., subgingival irrigation) and/or by controlled release delivery directly into the periodontal pocket using methods well known to the art (see, e.g., Kornman, K., (1993) *J. Periodontol.* 64, 782-791). Preferably, an inhibitor is applied subgingivally or by controlled release delivery.

The prolyl peptidases, active analogs, fragments, and modifications thereof can be used in a method of reducing growth of bacteria *in vitro* or *in vivo*. Preferably, the bacteria is a periodontal pathogen, i.e, a bacterial pathogen that causes periodontal disease, more preferably the bacteria is *P. gingivalis*. The inability of asaccharolytic *P. gingivalis* to utilize free amino acids makes the bacterium entirely dependant on an external peptide supply. The action of the polypeptides of the invention may be required for bacterial growth, and inhibition of the polypeptides of the invention may inhibit the *in vivo* growth of organisms, including *P. gingivalis*. The method includes decreasing the amount of dipeptides and/or tripeptides (e.g., the result of cleavage of SEQ ID NO:25 by a prolyl-tripeptidyl peptidase) and the amount of free amino acids that result from further cleavage of the dipeptides and/or tripeptides present by inhibiting a prolyl

peptidase, active analog, active fragment, or active modification thereof, such that the amount of dipeptides and/or tripeptides generated by the polypeptides is decreased. The amount of dipeptides and/or tripeptides is decreased relative to the amount of dipeptides and/or tripeptides present in the absence of the inhibitor. Preferably, the amount of dipeptides and/or tripeptides generated is decreased by an inhibitor, a monoclonal antibody that inhibits the prolyl peptidase, or polyclonal antibodies that inhibit the prolyl peptidase, more preferably, the amount of dipeptides and/or tripeptides generated is decreased by an inhibitor. Preferably, an inhibitor acts to inhibit a polypeptide of the invention, preferably a prolyl peptidase, by blocking the active site of the polypeptide. The polypeptide can be present on the surface of the bacteria or secreted into the environment, preferably the polypeptide is present in the surface of the bacteria.

The present invention is also directed to a method of developing an inhibitor of a prolyl peptidase, active analog, active fragment, or active modification thereof, preferably a prolyl-tripeptidyl peptidase. The method includes identifying a molecule that inhibits the amidolytic activity of the prolyl peptidase. This can be accomplished by, for instance, incubating the prolyl peptidase with a candidate molecule under conditions that promote amidolytic activity of the prolyl peptidase and determining if the amidolytic activity of the prolyl peptidase is decreased relative to the amidolytic activity in the absence of the molecule. The amidolytic activity can be measured by cleavage of the Pro-Yaa peptide bond present in the target polypeptide SEQ ID NO:25 as described herein. One method of developing an inhibitor includes using the target peptide SEQ ID NO:25 and replacing the Xaa residues with modified amino acids. It is expected that some modified amino acids will cause the target peptide to act as an inhibitor.

EXAMPLES

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

Example 1

Materials

Diisopropylfluorophosphate (DFP), leupeptin and 3,4-dichloroisocoumarin, were purchased from Calbiochem (La Jolla, CA). Antipain, iodoacetamide, substance P, bradykinin and bradykinin related peptides, were obtained from Sigma. Other peptides used in this study were synthesized at the Molecular Genetic Instrumental Facility (University of Georgia, Athens, GA) using Fmoc protocol with an advanced ChemTech MPS350 automated synthesizer. H-Ala-Phe-Pro-pNA, H-Gly-Pro-pNA, Z-Gly-Pro-pNA, Z-Ala-Pro-pNA, and H-Pro-pNA (where pNA is p- Nitroanilide; Z is benzyloxycarbonyl; and H is hydrogen and denotes an unblocked amino-terminal group) were obtained from Bachem (King of Prussia, PA). Prolinal was kindly provided by Dr. James Powers (Georgia Institute of Technology, Atlanta) and cystatin C by Dr. Magnus Abrahamson (University of Lund, Sweden).

Methods

Source and Cultivation of Bacteria— *P. gingivalis* HG66 was obtained from Dr. Roland Arnold (University of North Carolina, Chapel Hill), while the strains W50 (ATCC 53978) and ATCC 33277 were obtained from the ATCC. All cells were grown as described previously (Chen, Z., et al., (1992) *J. Biol. Chem.* 267, 18896-18901).

Enzyme Activity Assays— Routinely, the tripeptidyl peptidase amidolytic activity was measured with H-Ala-Phe-Pro-pNA (1mM) in 0.2 M HEPES (N-2-hydroxyethylpeperazine,N'-2-ethansulfonic acid), pH 7.5 at 37°C. The concentration of enzyme was 0.1 nM to 1 nM. The assay was performed in a total volume of 0.2 ml on microplates, and the initial turnover rate was recorded at 405 nm using a microplate reader (Spectramax Molecular Devices, Sunnyvale, CA). In inhibition studies, the enzyme was first preincubated with inhibitor for 15 min at 37°C, substrate added, and residual activity recorded after 5 minutes to 30 minutes. H-Gly-Pro-pNA, Z-Ala-Pro-pNA, Z-Gly-Pro-pNA and H-Pro-pNA (1 mM final concentration) were assayed in the same manner.

Protein Determination—Protein concentration was determined using the BCA reagent kit (Sigma, St. Louis, MO), using bovine serum albumin as a standard.

Localization of Tripeptidyl-Peptidase Activity—Cultures of *P. gingivalis* HG66, W50 and ATCC 33277, at different phases of growth, were subjected to the following fractionation procedure. The cells were removed by centrifugation (10,000 x g, 30 minutes), washed twice with 10 mM Tris, 150 mM NaCl, pH 7.4, resuspended in 50 mM Tris, pH 7.6, and disintegrated by ultrasonication in an ice bath at 1500 Hz for 5 cycles (5 minutes sonication/5 minutes brake). Unbroken cells and large debris were removed by centrifugation (10,000 x g, 30 minutes) and the opalescent supernatant subjected to ultracentrifugation (150,000 x g, 120 minutes), yielding a pellet containing bacterial membranes and a supernatant which was considered as membrane-free cell extract. All fractions, as well as the full culture, culture medium, and full culture after sonication, were assayed for amidolytic activity against H-Ala-Phe-Pro-pNA.

Enzyme Purification—All purification steps were performed at 4°C except for FPLC separations, which were carried out at room temperature. Cells were harvested by centrifugation (6,000 x g, 30 minutes), washed with 50 mM potassium phosphate buffer, pH 7.4, and resuspended in the same buffer (150 ml per 50 gram of cells wet weight). Triton X-100 (10% volume/volume in H_2O) was added slowly to the bacterial cell suspension to a final concentration of 0.05%. After 120 minutes of gentle stirring, unbroken cells were removed by centrifugation (28,000 x g, 60 minutes). Proteins in the supernatant were precipitated with cold acetone (-20°C) added to a final concentration of 60% and collected by centrifugation. The pellet was redissolved in 50 mM potassium phosphate buffer, pH 7.0, and extensively dialyzed against 20 mM potassium phosphate, pH 7.0, containing 0.02% sodium azide. The dialyzed fraction was clarified by centrifugation (28,000 x g, 30 min) and applied to a hydroxyapatite column (BioRad, Melville, NY) equilibrated with 20 mM potassium phosphate, pH 7.0, at a flow rate of 20 ml/hour. After equilibration, the column was washed until the A_{280} fell to zero. Bound proteins were eluted with a gradient from 20-300 mM potassium phosphate and fractions (7 ml) analyzed for dipeptidyl- and tripeptidyl-peptidase activity using H-Gly-Pro-pNA and H-Ala-Phe-Pro-pNA, respectively. The activity against the latter

substrate was pooled, saturated with 1 M ammonium sulfate, clarified by centrifugation, and directly loaded onto a Phenyl-Sepharose HP (Pharmacia, Piscataway, NJ) column equilibrated with 50 mM potassium phosphate, pH 7.0, containing 1 M ammonium sulfate. The column was washed with two volumes of equilibration buffer, followed by buffer containing 0.5 M ammonium sulfate, and developed with a descending gradient of ammonium sulfate from 0.5 to 0 M. Active fractions were pooled, extensively dialyzed against 20 mM Tris, pH 7.5, and applied to a MonoQ HR 5/5 FPLC column equilibrated with the same buffer. The column was washed with 5 volumes of equilibration buffer at 1.0 ml/minute, following which bound proteins were eluted with a gradient of 0- 300 mM NaCl. The active fractions were pooled, dialyzed against 25 mM Bis-Tris, pH 6.3, and subjected to chromatofocusing on a MonoP FPLC column equilibrated with Bis-Tris buffer, using a pH gradient developed with 50 ml of 10x diluted Polybuffer 74 (Pharmacia), adjusted to a pH of 4.0.

Electrophoretic Techniques— The SDS-PAGE system of Schagger and von Jagow (Schagger, H., and von Jagow, G. (1987) *Anal. Biochem.* 166, 368-379), was used to monitor enzyme purification and estimate the enzyme molecular mass. For amino-terminal sequence analysis, proteins resolved in SDS-PAGE were electroblotted to polyvinylidene difluoride membranes using 10 mM CAPS, pH 11, 10% methanol (Matsudaira, P. (1987) *J. Biol. Chem.* 262, 10035-10038). The membrane was washed thoroughly with water and stained with Coomasie Blue G250. The blot was air dried, and protein bands cut out and subjected to NH_2-terminal sequence analysis with an Applied Biosystems 491 Protein Sequencer using the program designed by the manufacturers.

Enzyme Fragmentation —The purified prolyl tripeptidyl peptidase (PTP-A) was partially denatured by incubation in 6 M urea in 0.02 M Tris, pH 7.6, for 60 minutes. Low molecular mass gingipain R (RgpB) (Potempa, J., et al. (1995) *Prospect. Drug Discovery and Design* 2, 445-458) from *P. gingivalis* was then added to make an enzyme:substrate molar ratio of 1:100. The reaction mixture was made in 1 mM cysteine and the sample incubated overnight at 37°C. Generated peptides were separated by reverse-phase HPLC using a μBondapak C-18 column (3.9 x 300 mm) (Waters, Millford, MA). Peptides were eluted with 0.1% trifluoroacetic acid and acetonitrile containing 0.08% trifluoroacetic acid, using a

gradient from 0 to 80% acetonitrile over 60 minutes. Peptides were monitored at 220 nm and collected manually.

For determination of the active site serine residue and to confirm that the purified enzyme was a serine peptidase, 100 μg of purified PTP-A was first incubated with 170 μCi of [1,3-^{3}H]DFP (Amersham, Arlington Heights, IL) for 30 minutes at 25°C in 20 mM HEPES, pH 7.5. The reaction was quenched by addition of cold DFP to a final concentration of 10 mM and the radiolabelled material analyzed by SDS-PAGE, followed by autoradiographic analysis. The gel was dehydrated, soaked in PPO solution for 2 hours, dried, and the DFP-binding proteins detected by fluorography after an exposure time of 96 hours on X-ray film (XAR; Kodak, Rochester, NY). The bulk of radiolabelled protein was subjected to proteolytic fragmentation with RgpB and peptides obtained separated by reverse-phase HPLC as described above. Radioactivity in each peptide fraction was measured using a β liquid scintillation counter, and the labeled peptide, as well as other selected peptides were subjected to sequence analysis.

Identification of the PTP-A Gene— The database containing the unfinished *P. gingivalis* W83 genome, available from The Institute for Genomic Research, was searched for the presence of nucleotide sequences corresponding to the NH_2-terminal and the internal PTP-A amino acid sequences using the TBLASTN algorithm, BLAST version 2.0.8, and the default values for all parameters (Altschul, S.F., et al.,(1997) *Nucleic Acid Res.* 25, 3389-3402). An identified clone gnl | TIGR | P. gingivalis_126 was retrieved from The Institute for Genomic Research data base (http://www.tigr.org). The position of the PTP-A gene was localized using the NCBI open reading frame (ORF) finder (available from the National Center for Biotechnology Information, at http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The amino acid sequence, obtained by conceptual translation of the entire ORF, was further used for homology screening by use of the NCBI BLAST search tool.

Enzyme Specificity— Peptides were incubated with 1 μg PTP-A at an enzyme:substrate molar ratio of 1:100 for 3 hours or 24 hours in 50 μl of 200 mM HEPES, pH 7.5, at 37°C, and the reaction stopped by acidification with trifluoroacetic acid. The samples were then subjected to reverse-phase high

pressure liquid chromatography using a μBondapak C-18 column (3.9 x 300 mm) (Waters, Millford, MA) and an acetonitrile gradient (0-80 % in 0.075% trifluoroacetic acid in 50 min). Each peak, detected at 220 nm, was collected, lyophilized, re-dissolved in 50% (volume/volume) methanol, 0.1% acetic acid and subjected to analysis by mass spectrometry.

Mass Spectrometry—A Finnigan MAT 95S, sector mass spectrometer (Finnigan MAT, Bremen, Germany) equipped with an electrospray source (ESI) was used operated essentially as described previously (Stenfors, C., et al., (1997) *J. Biol. Chem.* 272, 5747-5751). Peptides were identified by fitting of the obtained spectra to specific sequences using an Internet application program MsFit available at http://falcon.ludwig.ucl.ac.uk/msfit.html.

Example 2

Enzyme Localization, Purification and Initial Characterization

Analysis of amidolytic activity against H-Ala-Phe-Pro-pNA in several fractions of *P. gingivalis* HG66, W50 and ATCC 33277 clearly indicated that an enzyme(s) with prolyl tripeptidyl-peptidase activity is localized on the cell surface in all strains tested with less than 5% of the total activity being found in the medium regardless of the growth phase of the bacterial culture. Cell associated enzyme was easily detached from the bacterial surface by treatment with a low concentration (0.05%) of Triton X-100. This procedure released more than 85-90% of activity in a soluble form. Subsequent acetone precipitation of proteins in the Triton X-100 fraction successfully separated the activity from pigment which remained in solution. The redissolved protein fraction, after dialysis, was applied to hydroxyapatite (100 ml) equilibrated with 20 mM potassium phosphate buffer pH 7.0. The elution was carried out with 20 mM potassium phosphate buffer pH 7.0, using a phosphate gradient from 20 mM to 300 mM at flow rate 20 ml/h. At this step substantial separation of the PTP-A activity from both the DPP IV and bulk protein was achieved (Fig. 1a). Further purification performed by subsequent chromatography steps including Phenyl-Sepharose (Fig. 1b), MonoQ (Fig. 1c) and MonoP columns (Fig. 1d), resulted in the isolation of purified enzyme.

Phenyl-Sepharose HP (25 ml) was equilibrated with 50 mM potassium phosphate, 1M ammonium sulfate, pH 7.0, at flow rate 30 ml/h. The column was

washed with two volumes of equilibration buffer and a step gradient of 0.5 M ammonium sulfate was applied, following which a descending gradient of 0.5 to 0 M ammonium sulfate was applied. The PTP-A containing fractions were extensively dialyzed against 20 mM Tris-HCl, pH 7.0, and concentrated by ultrafiltration. The concentrated PTP-A containing fractions were applied to a MonoQ column equilibrated with the same buffer. The column was washed with 5 volumes of equilibration buffer, following which bound protein was eluted with a gradient of 0-300 mM NaCl. The concentrated fraction of PTP-A from the MonoQ column was equilibrated with 25 mM Bis-Tris, pH 6.3, and loaded on a MonoP column equilibrated with the same buffer. A pH gradient was developed using 50 ml of Polybuffer 74, with the pH adjusted to 4.0.

Significantly, the chromatography step on the MonoP column yielded the A_{280} profile much sharper than the activity peak. Although this imperfect overlap of protein and activity may suggest that the protein component does not represent the active enzyme, the rest of data argues with such a contention. This apparent contradiction may be likely explained by the enzyme inhibition by the reaction product of H-Ala-Phe-Pro-pNA hydrolysis but this possibility has not been explored. The yield of protein and activity recovery in a typical purification procedure is summarized in Table 1.

Table 1. Purification of the PTP- A from *P. gingivalis*

Step	Volume (ml)	Protein (mg)	Total activity*	Specific activity (units/mg)	Purification fold	Yield (%)
Triton X-100 extract after centrifugation	200	1200	757 673	642	1	100
Acetone precipitate	50	600	537 622	896	1.4	71
Hydroxyapatite chromatography	50	22	400 039	18 183	28	53
Phenyl-Sepharose	48	10	312 505	31 250	48	41
MonoQ	3	1.5	244 828	163 218	254	32
MonoP	4	0.7	188 400	269 142	420	25

* Based on the enzymatic activity using H-Ala-Phe-Pro-pNA where one unit = mOD/min/1ml

SDS-PAGE analysis of the purified enzyme revealed the presence of two protein bands with apparent molecular masses of 81.8 and 75.8 kDa, respectively (Fig. 2, lane f). Autoradiography of the enzyme sample radiolabeled with [1,3-^{3}H]DFP (Fig. 2, lane g) clearly indicated that the bands represented either two distinct serine peptidases or different molecular mass forms of the same enzyme. In an attempt to distinguish between these two options, the electrophoretically resolved proteins were subjected to amino terminal sequence analysis. Unfortunately, it was found that the 81.8 kDa form of PTP-A had a blocked N-terminus. In contrast, the sequence NH_2-SAQTTRFSAADLNALMP (SEQ ID NO:23) was found at the N-terminus of the lower molecular mass form of the enzyme. This result led us to the possibility that the 75.8 kDa form of PTP-A was derived from the 81.8 kDa form through proteolytic cleavage of a 6 kDa amino-terminal peptide. To confirm this hypothesis and, in addition, to localize the active site residue within *P. gingivalis* PTP-A, the mixture containing both radiolabeled enzymes was proteolytically fragmented and peptides resolved by reverse-phase HPLC. This procedure yielded only one major radioactive peptide peak, and the purified peptide was found to have a single amino acid sequence: IGVHGWXYGGFMTTNL (SEQ ID NO:24), where X apparently represents the active-site serine residue covalently and irreversibly modified by DFP. These data convincingly indicate that the two protein bands of purified PTP-A represents different forms of the same enzyme. The portion of the purified PTP-A having a truncated N-terminus may be due to cleavage by Lys-specific peptidase and is likely to be an artifact which occurred during the purification procedure. Nevertheless, the proteolytic shedding of membrane bound PTP-A also occurs during cultivation of the bacteria, as indicated by variable amount of soluble activities found in cell free culture media.

Example 3

pH Optimum, Stability and Inhibition Profile

Using the amidolytic activity assay with H-Ala-Phe-Pro-pNA it was found that the enzyme has a broad pH optimum from pH 6.0 to 8.0 and in 0.2 M HEPES, pH 7.6 was stable for at least 12 hours at 25°C or 37°C. PTP-A activity was not affected by class specific synthetic inhibitors of cysteine or metalloproteinases

(Table 2). In contrast, preincubation of the enzyme with DFP or PEFABLOCK resulted in total loss of activity, supporting its classification as a serine peptidase. Surprisingly, however, 3,4-dichloroisocumarin was only a poor inhibitor, and PMSF, leupeptin, antipain and prolinal had no effect at all. Interestingly, preincubation of PTP-A with iodoacetamide, but not with N-ethylmaleimide, stimulated enzyme amidolytic activity about two-fold. Human plasma inhibitors, such as α_1-proteinase inhibitor, α_1-antichymotrypsin and α_2-macroglobulin did not affect the enzyme activity, nor were they cleaved by PTP-A.

The effect of inhibitors on amidolytic activity of DPP IV was also determined using the same conditions as those used for PTP-A, but using H-Gly-Pro-pNA as a substrate.

Table 2. Effect of inhibitors on the amidolytic activity of PTP-A and DPP IV. Results are for a 15-min incubation at 37 C in 0.2 HEPES pH 7.6, with 1 mM H-Ala-Phe-Pro-pNA as substrate.

Inhibitor	Concentration	Residual activity of PTP-A, %	Residual activity of DPP IV, %
Diisopropyl fluorophospate	10 mM	0	0
	10 mM	96	20
Phenylmethanesulfonyl fluoride	1mg/ml	20	15
	10mg/ml	0	0
PEFABLOC SC	1 mM	56	100
3,4-dichloroisocoumarin	5mM	200	100
Iodoacetamide	5 mM	100	100
N-Ethylmaleimide	1 mM	98	100
1,10-orthophenanthroline	5 mM	93	100
	0.1 mM	100	100
EDTA	0.1 mM	100	100
Leupeptin	0.1 mM	100	20
Antipain	10 mM	100	0
Prolinal	10 mM	100	30
Val-Pro	10 mM	100	1
Ala-Pro			
Ala-Gly-Pro			

Example 4

Substrate Specificity

Among several chromogenic substrates tested, including H-Ala-Phe-Pro-pNA, H-Gly-Pro-pNA, Z-Gly-Pro-pNA, Z-Ala-Pro-pNA, H-Pro-pNA, only H-Ala-Phe-Pro-pNA was hydrolyzed by PTP-A indicating a prolyl specific tripeptidyl-peptidase activity. To further confirm this specificity several synthetic peptides composed of 5 to 34 amino acid residues and containing at least one proline residue were tested as substrates for PTP-A. Out of 22 peptides tested only those with a proline residue in the third position from the amino terminal end were cleaved (Table 3), with the significant exception of peptides with adjacent proline residues (peptides 3, 4 and 16). In addition, a free α-amino group was absolutely required for cleavage after the third proline residue as exemplified by resistance to enzymatic hydrolysis of peptide 9, which differs from the peptide 8 only in acylation of the α-amino group of the N-terminal valine residue. Except for these two limitations, the peptide bond -Pro-↓-Yaa- was cleaved at the same rate in all peptides with the general formula NH_2-Xaa-Xaa-Pro-Yaa-$(Xaa)_n$ (SEQ ID NO:25), where Xaa represents any amino acid residue while Yaa could be any residue except proline, regardless of the chemical nature of the amino acids and the length of the peptide. In all cases the reaction was completed within 3 hours and prolonged incubation for 24 hours did not affect the pattern of cleavage, confirming the absolute requirement for a proline residue at the third position from the unblocked N-terminus. In addition, these data indicate that the preparation of PTP-A was free of any contamination with either aminopeptidase, dipeptidyl peptidase, or endopeptidase activities.

The cleavage specificity of DPP IV was also determined using the same conditions as those used for PTP-A. The results (Table 3) demonstrate that DPP IV does not cleave between two proline residues.

Table 3. Cleavage specificity of PTP-A and DPP IVon synthetic peptides.

Substrate	Cleavage site	SEQ ID NO:
Peptide 1	H-Arg-Pro-**Pro**-↓-Gly-Phe-Ser-**Pro**-Phe-Arg	1
Peptide 2	H-Arg-**Pro**-**Pro**-↓-Gly-Phe	2
Peptide 3	H-Lys-Arg-**Pro**-**Pro**-Gly-Phe-Ser-**Pro**-Phe-Arg	3
Peptide 4	H-Tyr-Arg-**Pro**-**Pro**-Gly-Phe-Ser-**Pro**-Phe-Arg	4
Peptide 5	H-Arg-**Pro**-**Hyp**-Gly-Phe-Ser-**Pro**-Phe-Arg	5
Peptide 6	H-Arg-**Pro**-↑-Lys-**Pro**-↑-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2	6
Peptide 7	H-Val-**Pro**-**Pro**-↓-Gly-Glu-Asp-Ser-Lys-Glu-Val-Ala-Ala-**Pro**-His-Arg-Gln	7
Peptide 8	H-Val-**Pro**-**Pro**-↓-Gly-Glu-Asp-Ser-Lys	8
Peptide 9	Ac-Val-**Pro**-**Pro**-Gly-Glu-Asp-Ser-Lys	9
Peptide 10	H-Val-Glu-**Pro**-↓-Ile-**Pro**-Tyr	10
Peptide 11	H-Arg-Gly-**Pro**-↓-Phe-**Pro**-Ile	11
Peptide 12	H-Ala-Arg-**Pro**-↓-Ala-D-Lys-amide	
Peptide 13	H-**Pro**-Asn-**Pro**-↓-**Asn**-Gln-Gly-Asn-Phe-Ile	13
Peptide 14	H-Arg-His-**Pro**-↓-Lys-Tyr-Lys-Thr-Glu-Leu	14
Peptide 15	H-Gly-Val-**Pro**-↓-Lys-Thr-His-Leu-Glu-Leu	15
Peptide 16	H-Lys-Gly-**Pro**-**Pro**-Ala-Ala-Leu-Thr-Leu	16
Peptide 17	H-Gln-Lys-Gln-Met-Ser-Asp-Arg-Arg-Glu-Asn-Asp-Met-Ser-**Pro**-Ser-Asn-Asn-Val-Val-**Pro**-Ile-His-Val-**Pro**-**Pro**-Thr-Thr-Glu-Asn-Lys-**Pro**-Lys-Val-Gln	17
Peptide 18	H-Phe-Leu-Arg-Glu-**Pro**-Val-Ile-Phe-Leu	18
Peptide 19	H-Gly-Ile-Arg-**Pro**-Tyr-Glu-Ile-Leu-Ala	19
Peptide 20	H-Leu-**Pro**-↑-Asp-Leu-Asp-Ser-Ser-Leu-Ala-Ser-Ile-Gln-Glu-Leu-Leu-Ser-**Pro**-Gln-Glu-**Pro**--**Pro**-Arg-**Pro**-**Pro**-Glu-Ala	20
Peptide 21	H-Cys-Leu-Ser-Ser-Gly-Thr-Leu-**Pro**-Gly-**Pro**-Gly-Asn-Asp-Ala-Ser-Arg-Glu-Leu-Glu-Ser	21
Peptide 22	H-Lys-Ile-**Ala**-Gly-Tyr-His-Leu-Glu-Leu	22
Peptide 23	H-Ser-**Pro**-↑-Tyr-Ser-Ser-Asp-Thr-Thr	46
Peptide 24	H-Ala-**Pro**-↑-Val-Arg-Ser-Leu-Asn-Cys-Thr-Leu-Arg-Asp-Ser-Gln-Gln-Lys	47

↓ indicates cleavage site mediated by PTP-A

↑ indicates cleavage site mediated by DPP IV

The lack of cleavage after internal proline residues in the synthetic peptides corresponds well with the absence of any proteolytic activity on several protein substrates including IgA, IgG, albumin, azocasein, carboxymethylated rybonuclease and gelatin. However, the size of substrate, which is a limiting factor in the activity of oligopeptidases (Walter, R., et al., (1980) *Mol. Cell. Biochem.* 30, 111-126), is not restricting in the case of PTP-A, because the enzyme is able to cleave a tripeptide (NH_2-Xaa-Xaa-Pro) from the N-terminus of both human cystatin C and interleukin 6.

Example 5

PTP-A Sequence Analysis

Partial PTP-A amino acid sequence data allowed us to identify the *P. gingivalis* genomic clone gnl | TIGR | P. gingivalis_126 in the Unfinished Microbial Genomes data base, TIGR. An ORF corresponding to the PTP-A amino acid sequence was found as indicated by the fact that all sequences of the PTP-A derived peptides obtained by the enzyme polypeptide fragmentation with RgpB were present in the protein primary structure inferred from the nucleotide sequence of the ORF. The 732 amino acid polypeptide with a calculated mass of 82, 266 Da was encoded in this ORF. The homology search performed using the NCBI TBLASTN tool against GenBank+EMBL+DDBJ+PDB databases and subsequent multiple sequence alignments using the ClustalW Multiple Sequence Alignment tool (Fig. 3) indicated that PTP-A is a new member of the prolyl oligopeptidase (S9) family of serine peptidases (Rawlings, N.D., et al., (1991) *Biochem. J.* 279, 907-908).

The sequence GXSXGG (SEQ ID NO:40) is a signature feature for the S9 family of serine peptidases. Within this large and diverse S9 family of evolutionary and functionally related enzymes both from prokaryotes and eukaryotes, PTP-A was most closely related to bacterial dipeptidyl peptidase IV (DPP IV) from *Flavobacterium meningosepticum*, *Xantomonas maltophilus*, and *P. gingivalis*, sharing 31.6%, 30.4%, and 28.5% amino acid sequence identity, respectively. Remarkably, the COOH-terminal region of the PTP-A molecule (residues 502 - 732) shows a significant similarity to the eukaryotic prolyl oligopeptidases with 34% and 33% identity to human DPP IV and mouse fibroblast activation protein (FAP), respectively (Fig. 3). This part of the molecule contains the amino acid

residues which encompass the catalytic triad in all characterized prolyl oligopeptidases, and from the multiple alignments with DPP IV of confirmed active site residues (Kabashima,T., et al., (1995) *Arch. Biochem. Biophys.* 320, 123-128) it is apparent that Ser-603, Asp-678 and His-710 represent the catalytic triad of PTP-A (Fig. 3). Such an inference is further supported by the direct labeling of Ser-603 by DFP. In addition, the computer assisted search for sequential motifs characteristic for transmembrane domains revealed the presence of such a putative region within the N-terminal sequences of PTP-A, with residues 5 to 25 most likely folded into a hydrophobic α-helix responsible for membrane anchoring of this enzyme.

In *P. gingivalis* PTP-A, as well as in DPP IV, all activities are cell surface associated, and it is conceivable that the enzymes are membrane anchored through putative signal sequences which are not cleaved but remain as a membrane spanning domain similar to other members of the prolyl oligopeptidase family. The cell surface localization of di- and tripeptidyl-peptidases suggests a putative physiological function in providing nutrients for growing bacterial cells. Here, the inability of asaccharolytic *P. gingivalis* to utilize free amino acids (Dashper, S.G., et al., *J. Dent Res.* 77 ,1133 (Abstract) (1988)) makes the bacterium entirely dependant on an external peptide supply. In this regard, DPP-IV and PTP-A activities are probably very important, if not indispensable, for bacterial growth.

This suggestion is strongly corroborated by the fact that the *P. gingivalis* genome contains three additional genes encoding peptidases homologous with DPP-IV and PTP-A and one related to aminopeptidase B. The peptidases homologous with DPP-IV and PTP-A are referred to as homologs H1 (SEQ ID NO:43), H2 (SEQ ID NO:44), and H3 (SEQ ID NO:45) (Fig. 6). If expressed, each gene product would probably have enzymatic activity because each has a well preserved catalytic triad (Fig. 4). In addition, all of these genes encode a putative signal peptide which may act in providing membrane-anchorage motifs.

Example 6

Influence of Proteinase Inhibitor on *P. gingivalis* Growth

To evaluate whether *P. gingivalis* growth was influenced by the presence of a peptidase inhibitor, *P. gingivalis* in logphase growth was diluted 1:5 into liquid media and incubated at 37°C. The cell density was monitored by measuring the optical density at 600 nm (OD_{600}). When the optical density began to increase, Pefabloc was added at 0.5 mg/ml or at 2.0 mg/ml. The control culture received no Pefabloc. The cultures receiving Pefabloc exhibited decreased growth (Figure 5). The peptidase inhibitor had to be added before the culture reached an OD_{600} of about 0.3 for the peptidase inhibitor to have an effect on growth.

The complete disclosures of all patents, patent applications, publications, and nucleic acid and protein database entries, including for example GenBank accession numbers and EMBL accession numbers, that are cited herein are hereby incorporated by reference as if individually incorporated. Various modifications and alterations of this invention will become apparent to those skilled in the art without departing from the scope and spirit of this invention, and it should be understood that this invention is not to be unduly limited to the illustrative embodiments set forth herein.

Sequence Listing Free Text

SEQ ID NOs:1-11:	Synthetic peptides
SEQ ID NO:12:	Target peptide
SEQ ID NOs:13-22:	Synthetic peptides
SEQ ID NO:23:	Amino-terminus of the lower molecular mass form of PTP-A.
SEQ ID NO:24:	Amino acid sequence present in PTP-A, where X apparently represents the active-site serine residue covalently and irreversibly modified by DFP.
SEQ ID NO:25:	Target peptide, where Xaa represents a natural or modified amino acid residue, Yaa represents a natural or modified amino acid residue except proline, and N is equal to or greater than 1.
SEQ ID NO:26:	Mouse fibroblast activation protein

SEQ ID NO:27:	Human DPP IV
SEQ ID NO:28:	DPP from *Flavobacterium meningosepticum*
SEQ ID NO:29:	DPP from *P. gingivalis*
SEQ ID NO:30:	*P. gingivalis* PTP-A
SEQ ID NO:31:	Portion of PTP-A
SEQ ID NO:32:	Portion of DPP from *P. gingivalis*
SEQ ID NO:33:	Portion of H1 homolog of *P. gingivalis* DPP
SEQ ID NO:34:	Portion of H2 homolog of *P. gingivalis* DPP
SEQ ID NO:35:	Portion of H3 homolog of *P. gingivalis* DPP
SEQ ID NOs:36-37:	Probes
SEQ ID NO:38:	Nucleotide sequence of coding region encoding PTP-A.
SEQ ID NO:39:	Consensus sequence for clan SC where X is any amino acid and S is the active site serine GXSXXG.
SEQ ID NO:40:	Consensus sequence for family S9 where X is any amino acid and S is the active site serine GXSXGG.
SEQ ID NO:41:	A specific substrate for a prolyl-tripeptidyl peptidase, where Xaa represents a natural or modified amino acid residue, and Yaa represents a natural or modified amino acid residue except proline.
SEQ ID NO:42:	DPP from *P. gingivalis*
SEQ ID NO:43:	H1 homolog of *P. gingivalis* DPP
SEQ ID NO:44:	H2 homolog of *P. gingivalis* DPP
SEQ ID NO:45:	H3 homolog of *P. gingivalis* DPP
SEQ ID NO:46:	Synthetic peptides
SEQ ID NO:47:	Synthetic peptides
SEQ ID NO:48:	Amino terminal sequence of DPP IV

What is claimed is:

1. An isolated prolyl tripeptidyl-peptidase, active analog, active fragment, or active modification thereof having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids, wherein the prolyl tripeptidyl-peptidase:target polypeptide ratio is at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours.

2. An isolated prolyl tripeptidyl-peptidase, active analog, active fragment, or active modification thereof having amidolytic activity at a prolyl tripeptidyl-peptidase:target polypeptide ratio of at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours, and wherein the prolyl tripeptidyl-peptidase is isolated from *P. gingivalis*.

3. The isolated prolyl tripeptidyl-peptidase of claim 2 wherein the peptide cleaved by the isolated prolyl tripeptidyl-peptidase comprises the sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:11, H-Ala-Arg-Pro-Ala-D-Lys-amide, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:25, and SEQ ID NO:37.

4. The isolated prolyl tripeptidyl-peptidase of claim 2 wherein the amino acid sequence of the isolated prolyl tripeptidyl-peptidase comprises the amino acid sequence GXSXXG (SEQ ID NO:39).

5. The isolated prolyl tripeptidyl-peptidase of claim 4 wherein the amino acid sequence of the isolated prolyl tripeptidyl-peptidase comprises the amino acid sequence GXSXGG (SEQ ID NO:40).

6. The isolated prolyl tripeptidyl-peptidase of claim 4 wherein the amino acid sequence of the isolated prolyl tripeptidyl-peptidase comprises SEQ ID NO:30.

7. An isolated polypeptide, active analog, active fragment, or active modification thereof having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids, wherein the polypeptide:target polypeptide ratio of at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours.

8. An isolated polypeptide comprising an amino acid sequence having a percentage amino acid identity of greater than 35 % with SEQ ID NO:30.

9. An isolated nucleic acid fragment encoding a prolyl-tripeptidyl peptidase, active analog, active fragment, or active modification thereof, having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids, wherein the prolyl tripeptidyl-peptidase:target polypeptide ratio of at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours.

10. The nucleic acid fragment of claim 9 wherein the nucleic acid fragment has a nucleotide sequence comprising SEQ ID NO:38.

11. The nucleic acid fragment of claim 9 wherein a complement of the nucleic acid fragment hybridizes to SEQ ID NO:38 under hybridization conditions of 0.5 M phosphate buffer, pH 7.2, 7 % SDS, 10 mM EDTA, at 68°C, followed by three for 20 minutes washes in 2x SSC, and 0.1 % SDS, at 65°C, wherein at least about 20 nucleotides of the complement hybridize.

12. An isolated nucleic acid fragment encoding a polypeptide wherein the polypeptide comprises an amino acid sequence having a percentage amino acid identity of greater than 35 % with SEQ ID NO:30.

13. A method of identifying an inhibitor of a prolyl-tripeptidyl peptidase, active analog, active fragment, or active modification thereof, comprising identifying a molecule that inhibits the amidolytic activity of the prolyl-

tripeptidyl peptidase by incubating the prolyl-tripeptidyl peptidase with the molecule under conditions that promote amidolytic activity of the prolyl-tripeptidyl peptidase and determining if the amidolytic activity of the prolyl-tripeptidyl peptidase is inhibited relative to the amidolytic activity in the absence of molecule.

14. A method of reducing growth of a bacterium comprising inhibiting a prolyl tripeptidyl-peptidase, active analog, active fragment, or active modification thereof, by contacting the prolyl tripeptidyl-peptidase with an inhibitor of the prolyl tripeptidyl-peptidase.

15. A method for protecting an animal from a periodontal disease caused by *P. gingivalis* comprising administering to the animal the inhibitor of claim 14 wherein the disease is selected from the group consisting of gingivitis and periodontitis.

16. The method of claim 15 wherein the inhibitor is administered by a method selected from the group consisting of subgingival application and controlled release delivery.

17. A method of reducing growth of a bacterium comprising inhibiting a prolyl dipeptidyl-peptidase, active analog, active fragment, or active modification thereof, by contacting the prolyl dipeptidyl-peptidase with an inhibitor of the prolyl dipeptidyl-peptidase.

18. An immunogenic composition comprising an isolated prolyl tripeptidyl-peptidase, or an antigenic analog, antigenic fragment, or antigenic modification thereof, the prolyl tripeptidyl-peptidase having amidolytic activity for cleavage of a peptide bond present in a target peptide having at least 4 amino acids, wherein the prolyl tripeptidyl-peptidase:target polypeptide ratio is at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours.

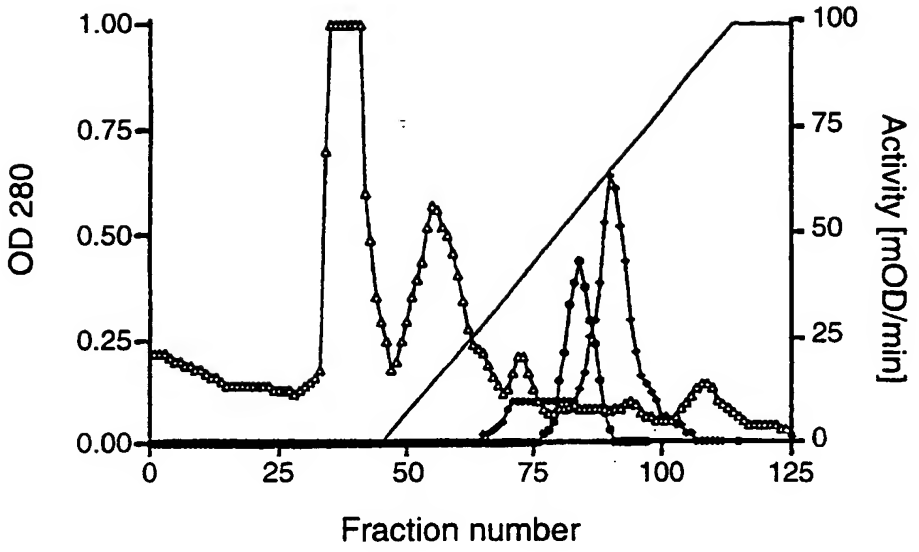
19. The immunogenic composition of claim 18 further comprising an adjuvant.

20. A composition comprising an inhibitor of an isolated prolyl tripeptidyl-peptidase and a pharmaceutically acceptable carrier.

21. A dipeptidyl peptidase having an amino acid sequence comprising SEQ ID NO:43.

22. A dipeptidyl peptidase having an amino acid sequence comprising SEQ ID NO:44.

23. A dipeptidyl peptidase having an amino acid sequence comprising SEQ ID NO:45.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0.75
0.50
0.25
0.00
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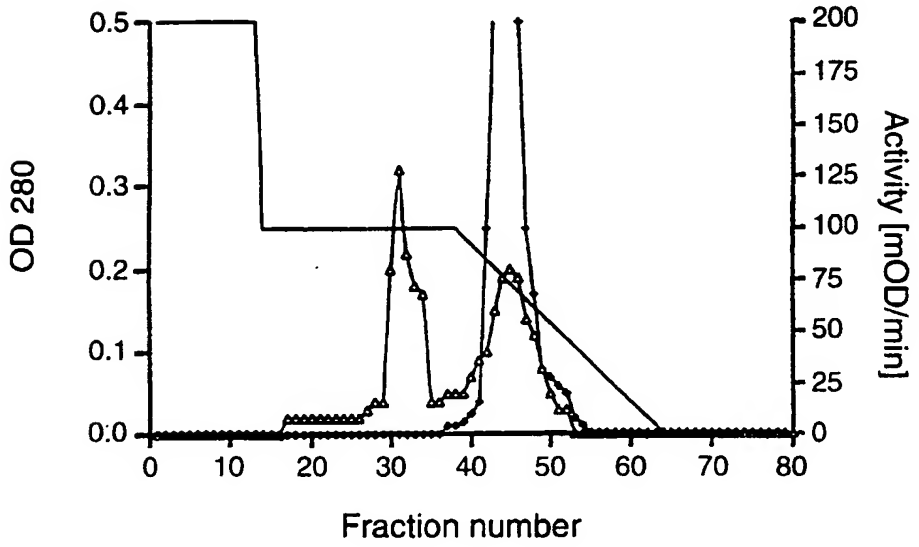
25
50
75
100
125
Fraction number
100
75
50
25
0
Activity [mOD/min]

Fig. 1a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0.4
0.3
0.2
0.1
0.0
OD 280
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10
20
30
40
50
60
70
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Fraction number
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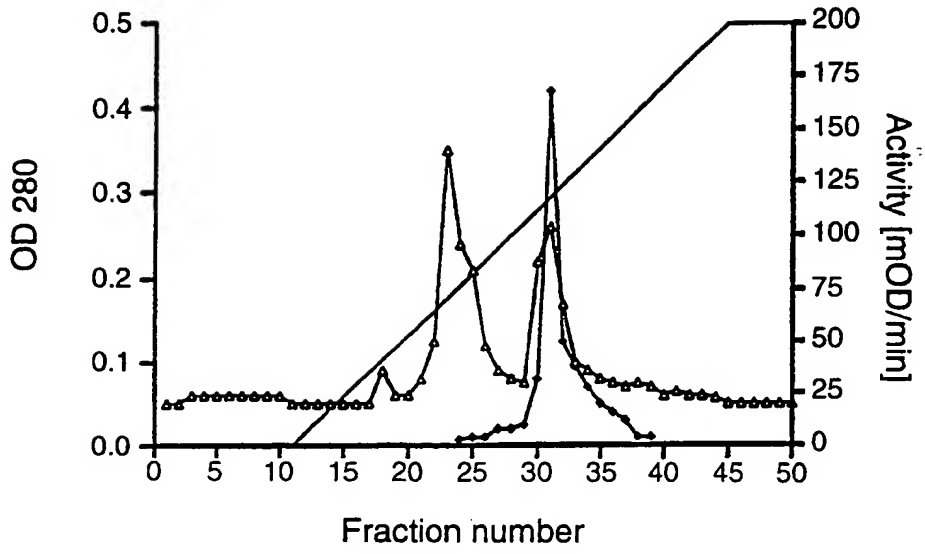
150
125
100
75
50
25
0
Activity [mOD/min]

Fig. 1b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Activity [mOD/min]
Fraction number
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100
75
50
25
0
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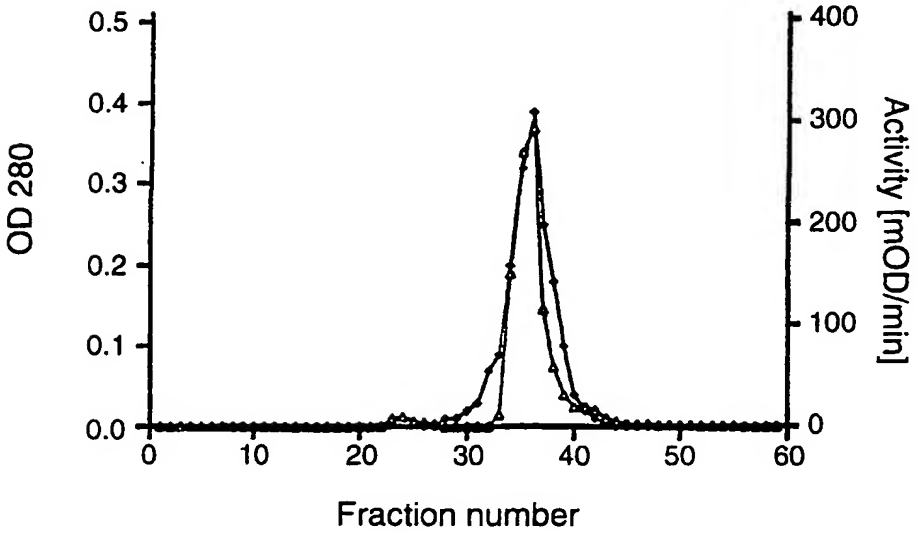
Fig. 1c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Activity [mOD/min]
Fraction number
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0.1
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0
0 10 20 30 40 50 60

Fig. 1d

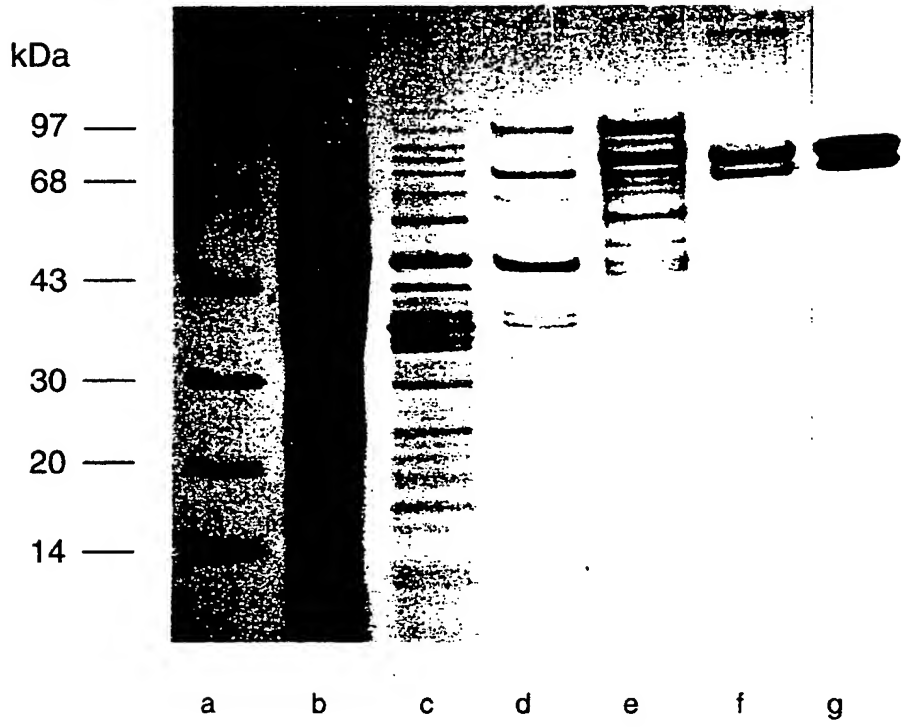

Fig. 2

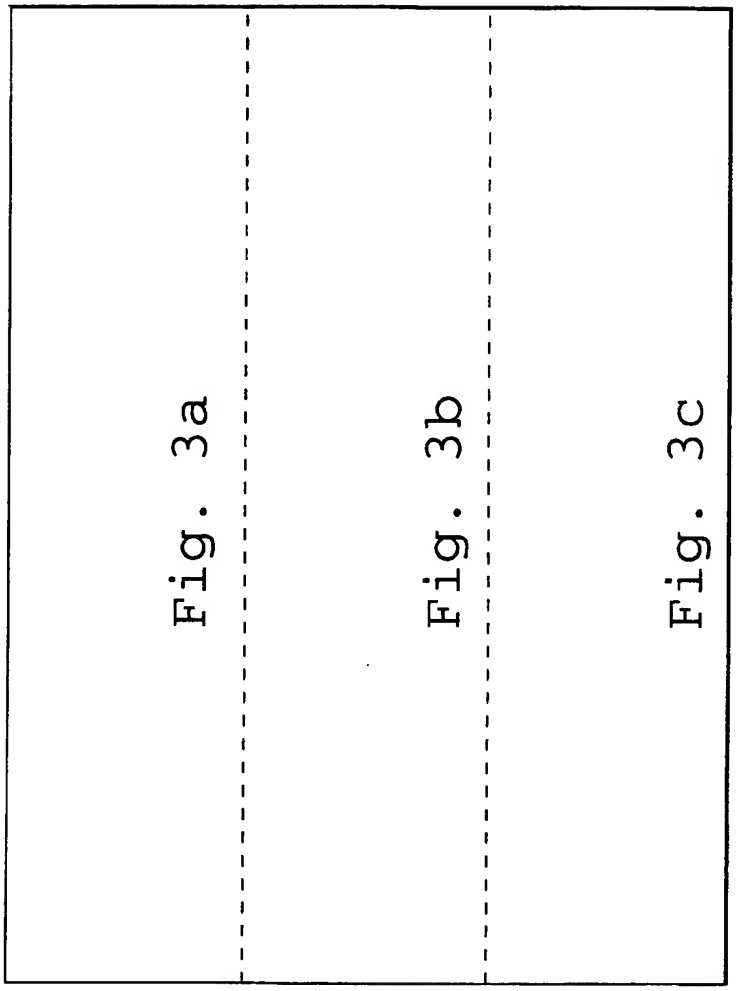

Fig. 3

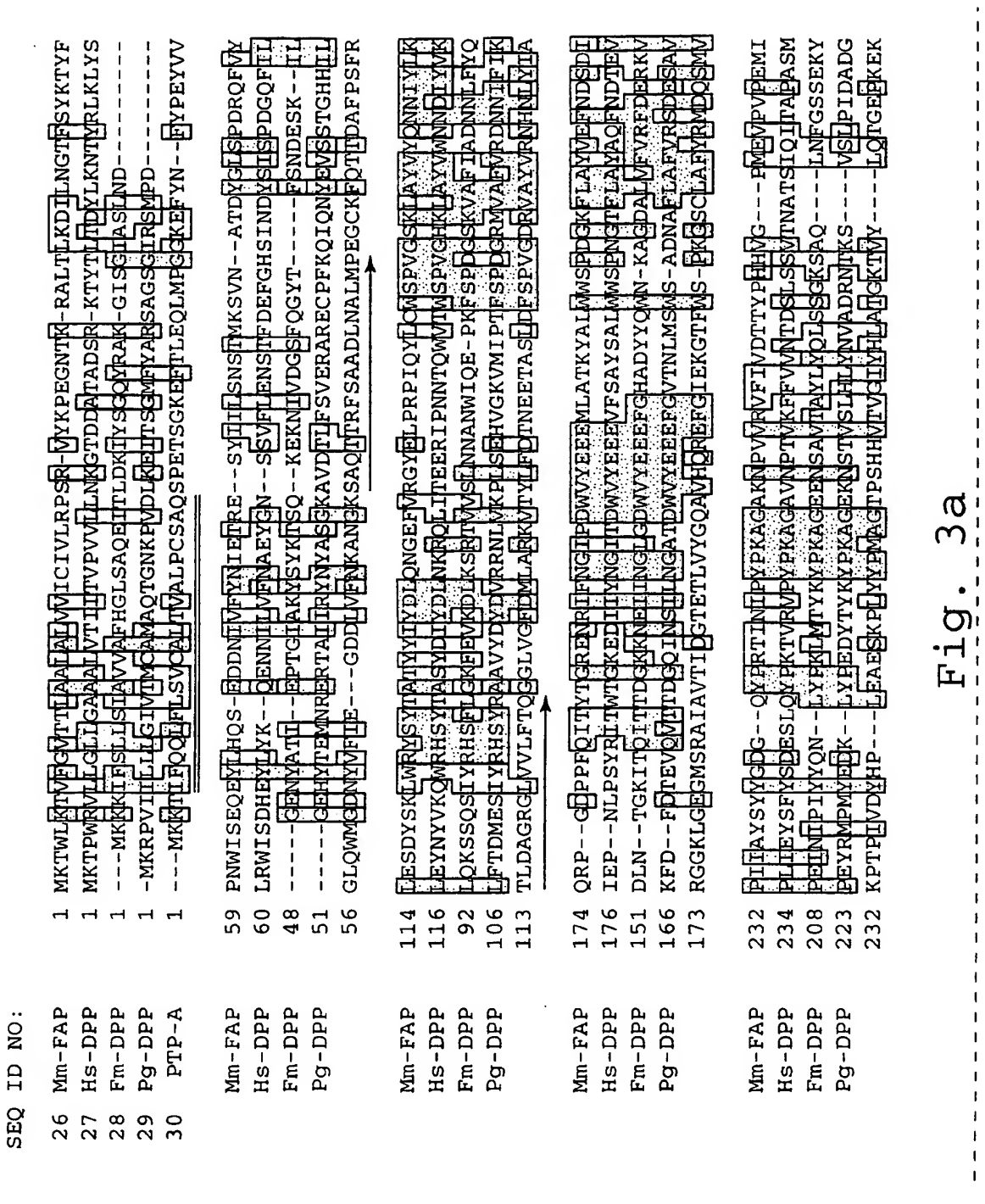

Fig. 3a

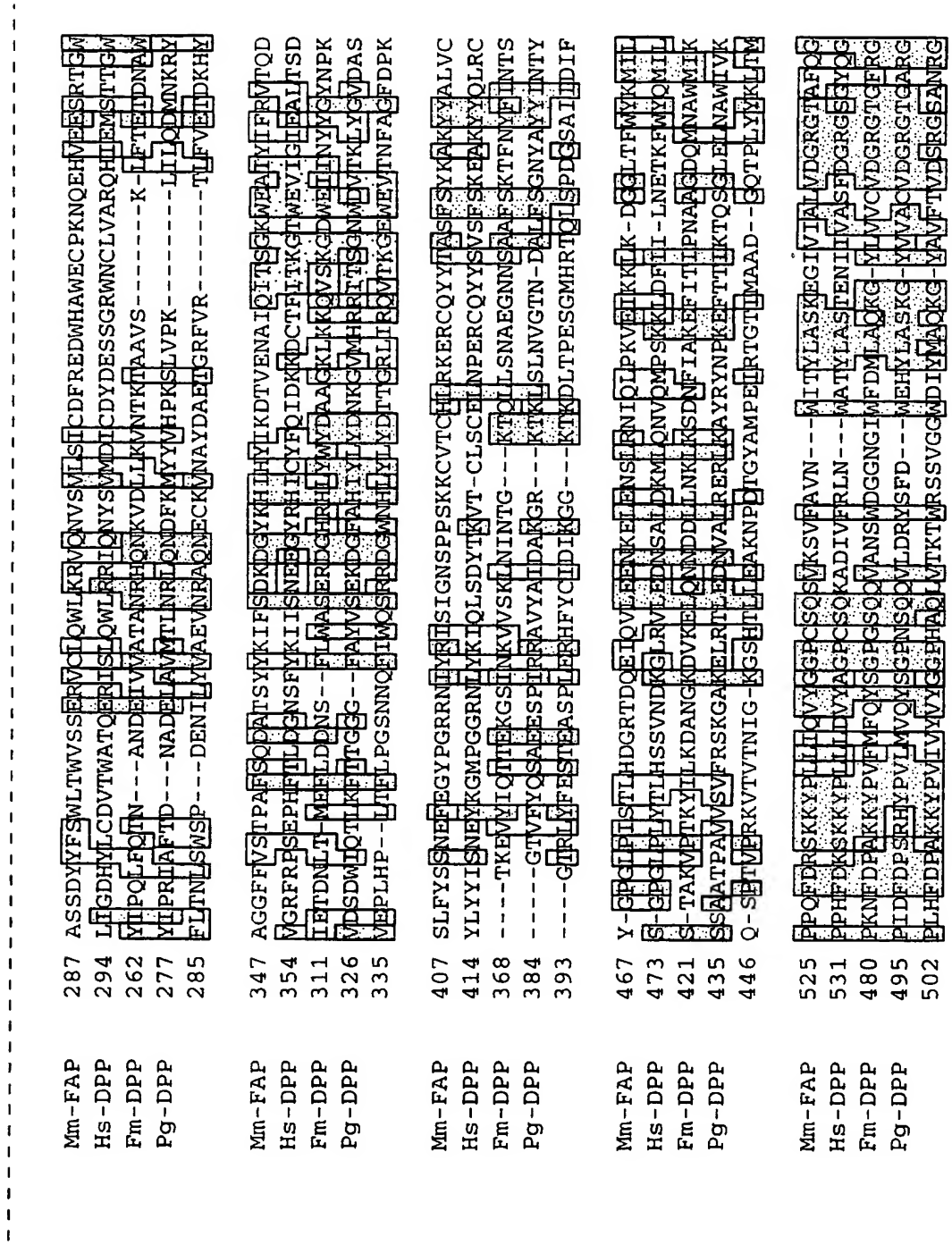

Fig. 3b

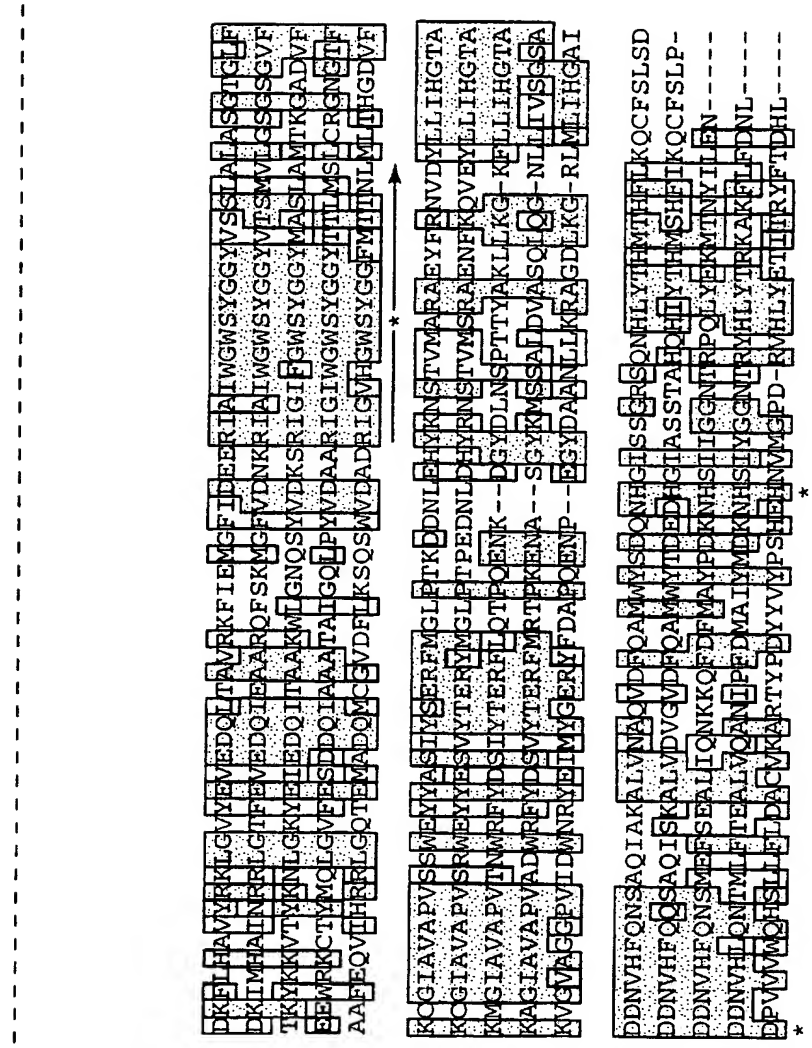

Fig. 3C

SEQ ID NO:
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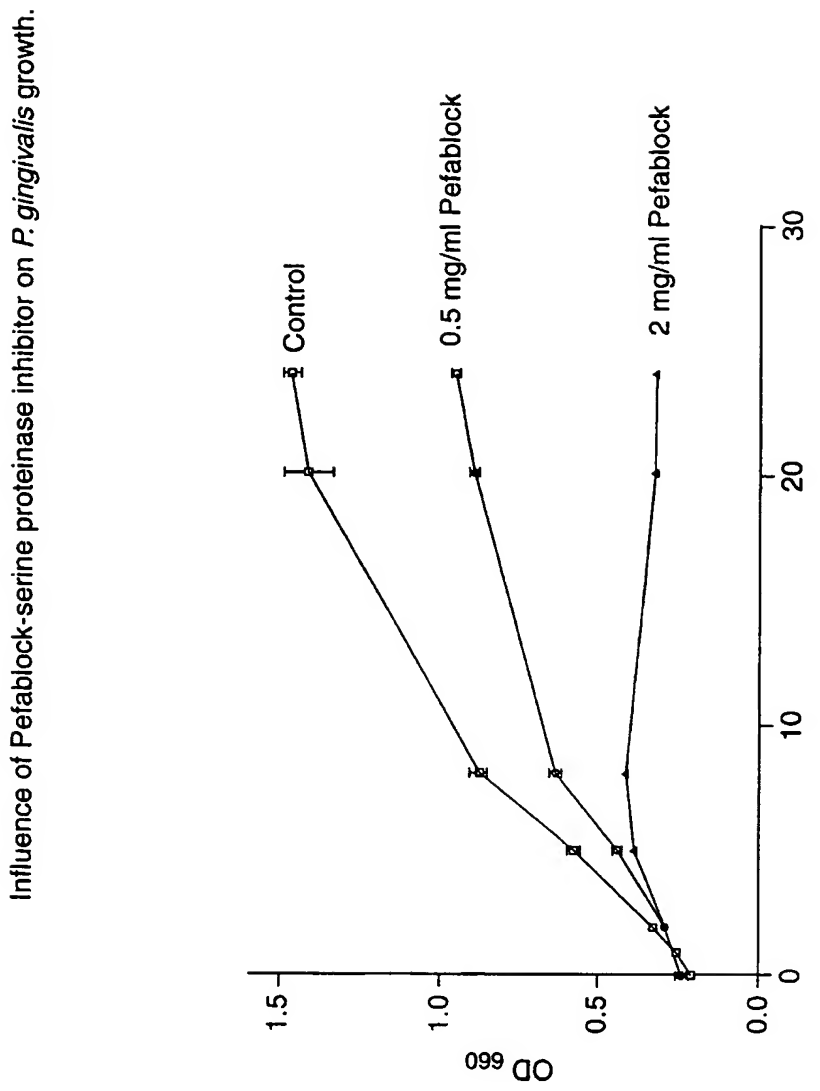
DPP 499 VDAARIGIWGWSYGGYTT 661 32
DPP-H1 350 VDPDRIAIYGASHGGYAT 524 33
DPP-H2 640 VNGKKVGGFGASYGGFMT 810 34
DPP-H3 495 VDGDRIGAVGASYGGFSV 667 35

Fig. 4


Influence of Pefablock-serine proteinase inhibitor on P. gingivalis growth.
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2 mg/ml Pefablock
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1.0
0.5
0.0
0
10
20
30
Time of growth [h]

Fig. 5

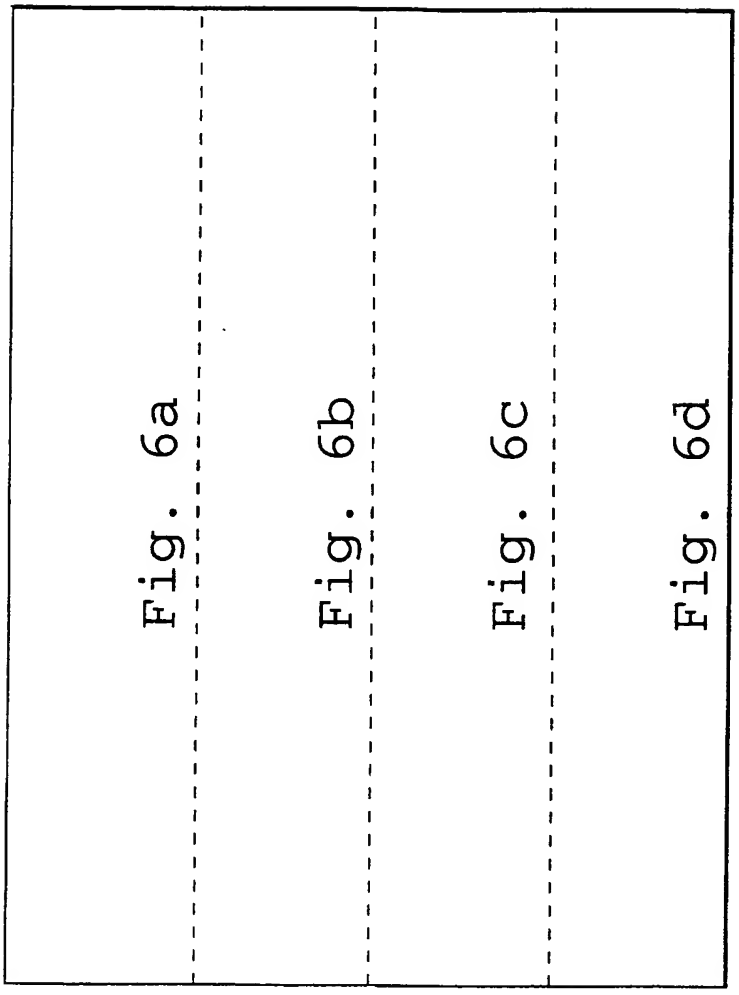

Fig. 6

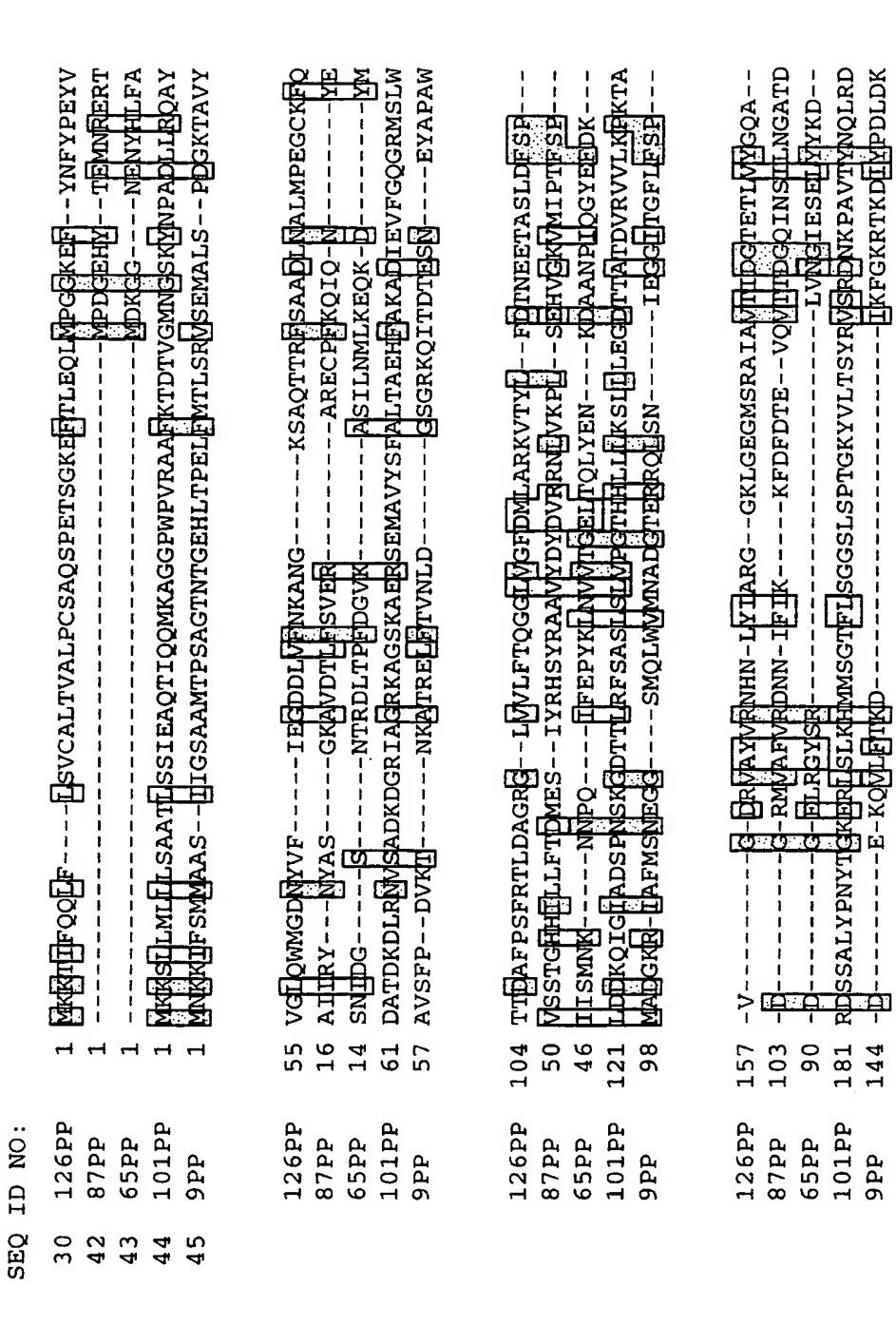

Fig. 6a

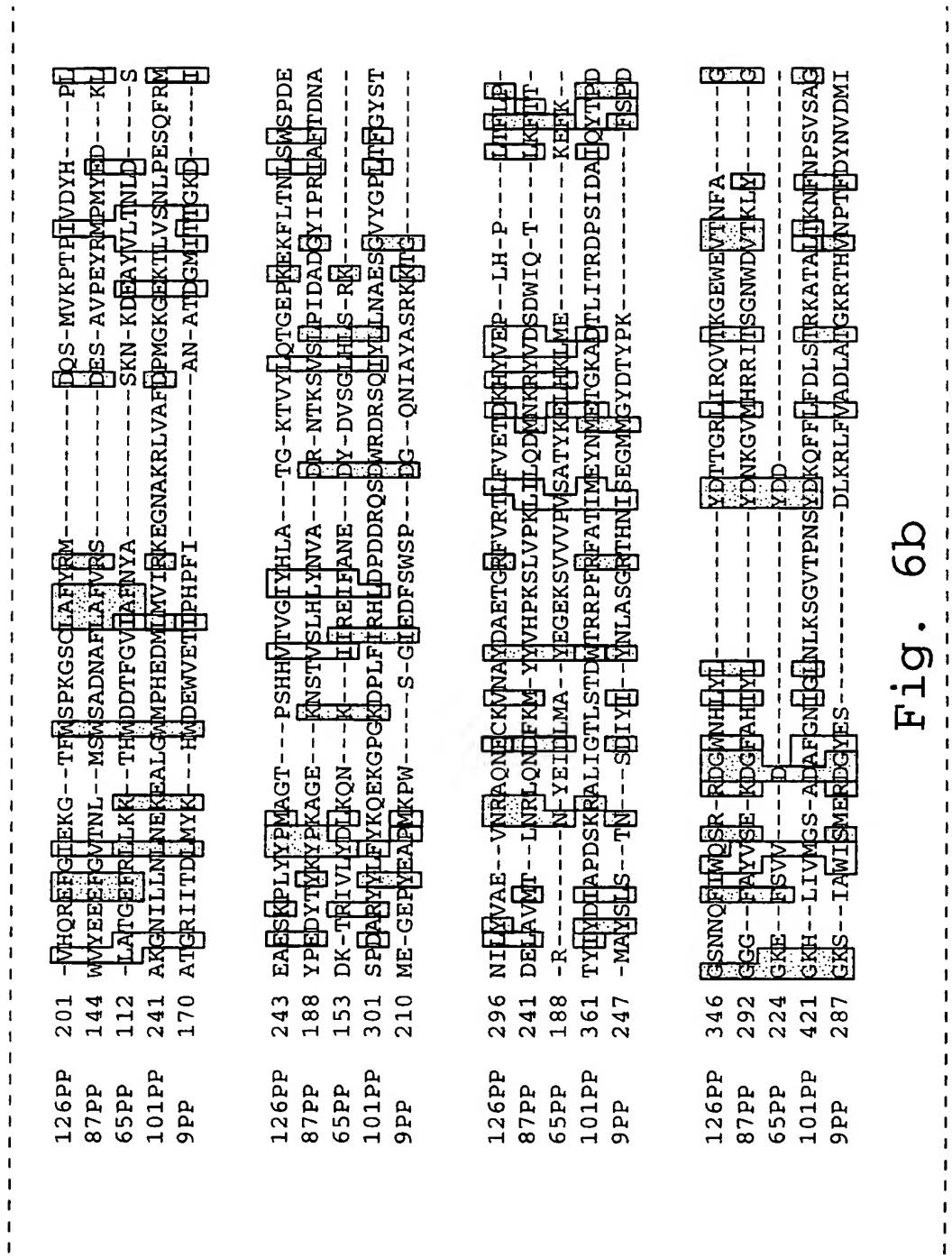

Fig. 6b

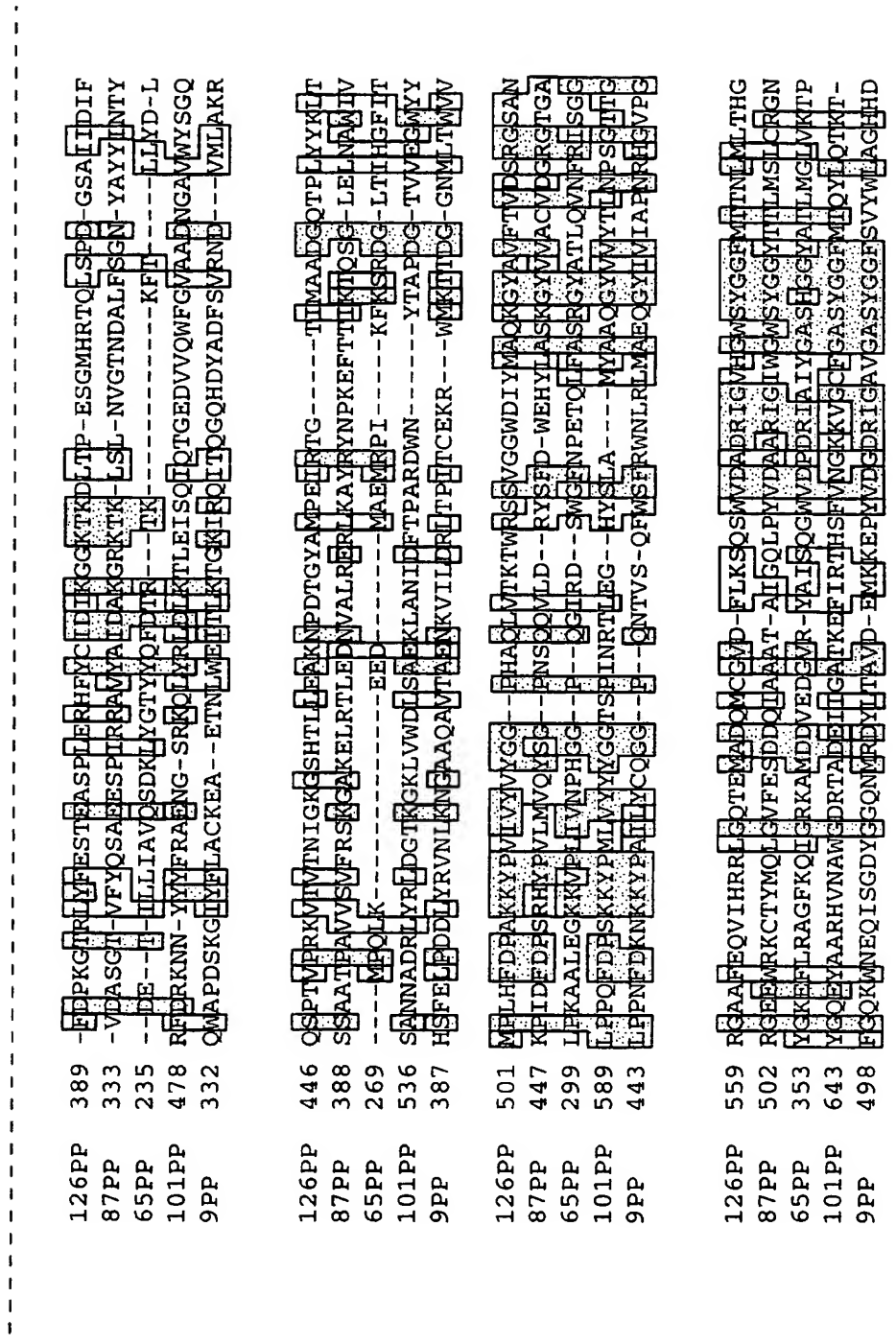

Fig. 6c

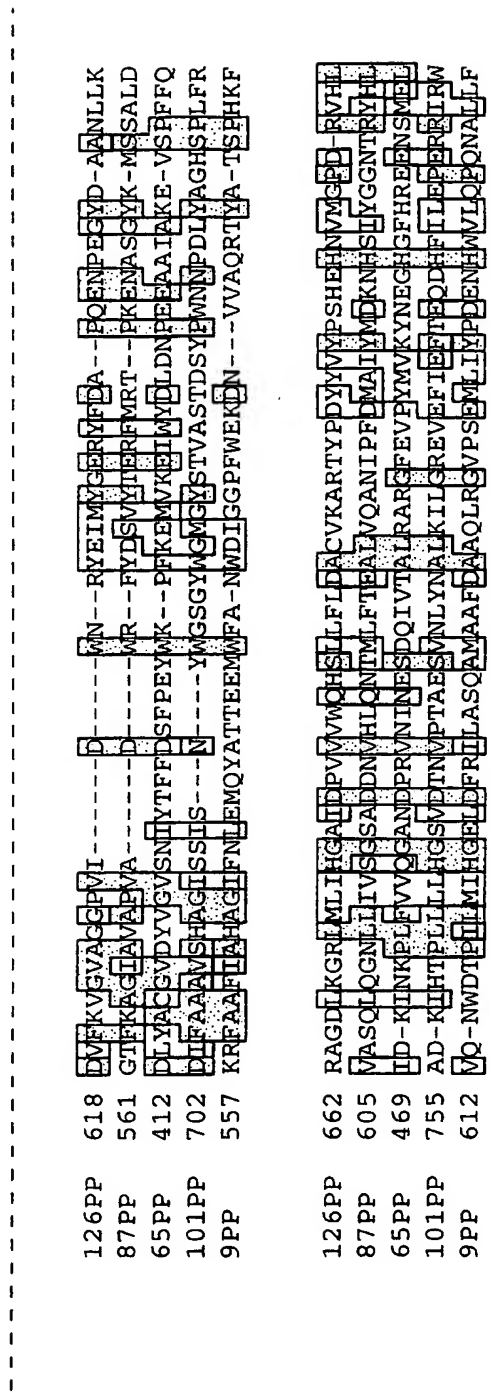

Fig. 6d

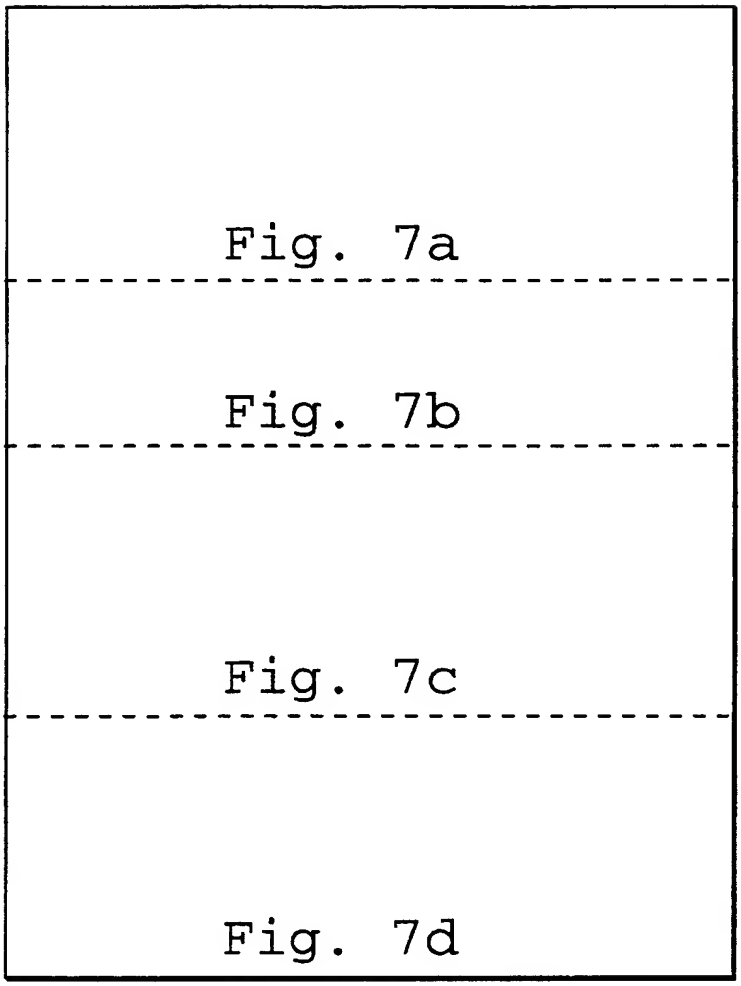

Fig. 7

P. gingivalis W 83 PTP sequence

SEQ ID NO: 38 13228 atgaagaagacaatcttccaacaactatttctgtctgtttgtgcc
SEQ ID NO: 30 M K K T I F Q Q L F L S V C A
13273 cttacagtggccttgccttgttcggctcagtctcctgaaacgagt
L T V A L P C S A Q S P E T S
13318 ggtaaggagtttactcttgagcaactgatgcccggaggaaaagag
G K E F T L E Q L M P G G K E
13363 ttttataacttttaccccgaatacgtggtcggtttgcaatggatg
F Y N F Y P E Y V V G L Q W M
13408 ggagacaattatgtctttatcgagggtgatgatttagtttttaat
G D N Y V F I E G D D L V F N
13453 aaggcgaatggcaaatcggctcagacgaccagattttctgctgcc
K A N G K S A Q T T R F S A A
13498 gatctcaatgcactcatgccggagggatgcaaatttcagacgact
D L N A L M P E G C K F Q T T
13543 gatgctttcccttcattccgcacactcgatgccggacggggactg
D A F P S F R T L D A G R G L
13588 gtcgttctatttacccaaggaggattagtcggattcgatatgctt
V V L F T Q G G L V G F D M L
13633 gctcgaaaggtgacttatcttttcgataccaatgaggagacggct
A R K V T Y L F D T N E E T A
13678 tctttggatttttctcctgtgggagaccgtgttgcctatgtcaga
S L D F S P V G D R V A Y V R
13723 aaccataacctttacattgctcgtggaggtaaattgggagaaggt
N H N L Y I A R G G K L G E G
13768 atgtcacgagctatcgctgtgactatcgatggaactgagactctc
M S R A I A V T I D G T E T L
13813 gtatatggccaggccgtacaccagcgtgaattcggtatcgaaaaa
V Y G Q A V H Q R E F G I E K
13858 ggtacattctggtctccaaaagggagctgccttgctttctatcga
G T F W S P K G S C L A F Y R
13903 atggatcagagtatggtgaagcctaccccgatagtggattatcat
M D Q S M V K P T P I V D Y H
13948 ccgctcgaagccgagtccaaaccgctttattaccccatggcaggt
P L E A E S K P L Y Y P M A G
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T P S H H V T V G I Y H L A T
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G K T V Y L Q T G E P K E K F
14083 ctgacgaatttgagttggagtccggacgaaaatatcttgtatgta
L T N L S W S P D E N I L Y V
14128 gctgaggtgaatcgtgctcaaaacgaatgtaaggtaaatgcctat
A E V N R A Q N E C K V N A Y
14173 gacgctgagaccggtagattcgtccgtacgctttttgttgaaacc
D A E T G R F V R T L F V E T
14218 gataaacattatgtagagccgttacatcccctgacattccttccg
D K H Y V E P L H P L T F L P

Fig. 7a

```
14263 ggaagtaacaatcagttcatttggcagagccgtcgcgacggatgg
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14308 aaccatctctatctgtatgatactacaggtcgtctgatccgtcag
      N  H  L  Y  L  Y  D  T  T  G  R  L  I  R  Q
14353 gtgacaaaaggggagtgggaggttacaaactttgcaggcttcgat
      V  T  K  G  E  W  E  V  T  N  F  A  G  F  D
14398 cccaagggaacacggctctatttcgaaagtaccgaagccagccct
      P  K  G  T  R  L  Y  F  E  S  T  E  A  S  P
14443 ctcgaacgccatttttactgtattgatatcaaaggaggaaagaca
      L  E  R  H  F  Y  C  I  D  I  K  G  G  K  T
14488 aaagatctgactccggagtcgggaatgcaccgcactcagctatct
      K  D  L  T  P  E  S  G  M  H  R  T  Q  L  S
14533 cctgatggttctgccataatcgatattttcagtcacctactgtc
      P  D  G  S  A  I  I  D  I  F  Q  S  P  T  V
14578 ccgcgtaaggttacagtgacaaatatcggcaaagggtctcacaca
      P  R  K  V  T  V  T  N  I  G  K  G  S  H  T
14623 ctcttggaggctaagaaccccgatacgggctatgccatgccggag
      L  L  E  A  K  N  P  D  T  G  Y  A  M  P  E
14668 atcagaacgggtaccatcatggcggccgatgggcagacacctctt
      I  R  T  G  T  I  M  A  A  D  G  Q  T  P  L
14713 tattacaagctcacgatgccgcttcatttcgatccggcaaagaaa
      Y  Y  K  L  T  M  P  L  H  F  D  P  A  K  K
14758 tatcctgttattgtctatgtttacggaggacctcatgcccaactc
      Y  P  V  I  V  Y  V  Y  G  G  P  H  A  Q  L
14803 gtaaccaagacatggcgcagctctgtcggtggatgggatatctat
      V  T  K  T  W  R  S  S  V  G  G  W  D  I  Y
14848 atggcacagaaaggctatgccgtctttacggtggatagtcgcgga
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14893 tctgccaatagaggggctgctttcgagcaggttattcatcgtcgt
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14938 ttggggcagaccgagatggccgatcagatgtgcggtgtggatttc
      L  G  Q  T  E  M  A  D  Q  M  C  G  V  D  F
14983 ctcaagagccaatcatgggtggatgccgatagaataggagtacat
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15028 ggctggagctatggtggctttatgactacgaatctgatgcttacg
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15073 cacggcgatgtcttcaaagtcggagtagccggcgggcctgtcata
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15118 gactggaatcgatatgagattatgtacggtgagcgttatttcgat
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15163 gcgccacaggaaaatcccgaaggatacgatgctgccaacctgctc
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15208 aaacgagccggtgatctgaaaggacgacttatgctgattcatgga
      K  R  A  G  D  L  K  G  R  L  M  L  I  H  G
15253 gcgatcgatccggtcgtggtatggcagcattcactccttttccttt
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Fig. 7b

```
      A  I  D  P  V  V  V  W  Q  H  S  L  L  F  L
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      D  A  C  V  K  A  R  T  Y  P  D  Y  Y  V  Y
15343 ccgagccacgaacataatgtgatggggccggacagagtacatttg
      P  S  H  E  H  N  V  M  G  P  D  R  V  H  L
15388 tatgaaacaataacccgttatttcacagatcacttatga 15426
      Y  E  T  I  T  R  Y  F  T  D  H  L  *
```

Fig. 7c

SEQ ID NO:38

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Fig. 7d

INTERNATIONAL SEARCH REPORT

Inte· ›nal Application No
PCT/US 00/05551

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/57 C12N9/48 A61K39/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KIYAMA, M. ET AL.: "Sequence analysis of the Porphyromonas gingivalis dipeptidyl peptidase IV gene" BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1396, no. 1, 4 March 1998 (1998-03-04), pages 39-46, XP000925951 cited in the application	7
A	the whole document	1-6, 8-16, 18-20

-/--

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 October 2000	10.01.01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fuchs, U

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 00/05551

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Derwent Publications Ltd., London, GB; Class B04, AN 1990-053917 XP002149298 & JP 02 005880 A (SUNSTAR KK), 10 January 1990 (1990-01-10)	7
A	abstract	1-6, 8-16, 18-20

P,X	BANBULA, A. ET AL.: "Prolyl Tripetidyl Peptidase from Porphyromonas gingivalis" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 14, April 1999 (1999-04), pages 9246-9252, XP002149297 the whole document	1-16, 18-20

A	KABASHIMA, T. ET AL.: "Cloning, Sequencing, and Expression of the Dipeptidyl Peptidase IV Gene from Flavobacterium meningosepticum in Escherichia coil" ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol. 320, no. 1, 20 June 1995 (1995-06-20), pages 123-128, XP000925965 the whole document	1-16, 18-20

A	KURAMITSU, H.K.: "Proteases of Porphyromonas gingivalis: what don't they do?" ORAL MICROBIOLOGY AND IMMUNOLOGY, vol. 13, no. 5, October 1998 (1998-10), pages 263-270, XP000925947 abstract page 267, column 2, line 34 -page 268, column 1, line 58	1-16, 18-20

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/05551

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 15 and 16 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-16 AND 18-20 COMPLETELY

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-16 and 18-20 completely

An isolated prolyl tripeptidyl peptidase, active analog, active fragment, or active modification thereof having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids; an isolated prolyl tripeptidyl peptidase, active analog, active fragment, or active modification thereof being isolated from Porphyromonas gingivalis; an isolated polypeptide, active analog, active fragment, or active modification thereof having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids; an isolated polypeptide comprising an amino acid sequence having a percentage amino acid identity of greater than 35% with SEO ID NO: 30; an isolated nucleic acid fragment encoding a prolyl tripeptidyl peptidase, active analog, active fragment, or active modification thereof having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids, an isolated nucleic acid fragment encoding a polypeptide comprising an amino acid sequence having a percentage amino acid identity of greater than 35% with SEQ ID NO: 30; a method of identifying an inhibitor of a prolyl tripeptidyl peptidase, active analog, active fragment, or active modification thereof; a method of reducing growth of a bacterium comprising inhibiting a prolyl tripeptidyl peptidase, active analog, active fragment, or active modification thereof; an immunogenic composition comprising an isolated prolyl tripeptidyl peptidase, or an antigenic analog, antigenic fragment, or antigenic modification thereof, the prolyl tripeptidyl peptidase having amidolytic activity for cleavage of a peptide bond present in a target peptide having at least 4 amino acids; a composition comprising an inhibitor of an isolated prolyl tripeptidyl peptidase;

2. Claim : 17 partially and 21 completely

A method of reducing growth of a bacterium comprising inhibiting a prolyl dipeptidyl peptidase, active analog, active fragment, or active modification thereof; a dipeptidyl peptidase having an amino acid sequence comprising SEQ ID NO: 43;

3. Claim : 17 partially and 22 completely

A method of reducing growth of a bacterium comprising inhibiting a prolyl dipeptidyl peptidase, active analog, active fragment, or active modification thereof; a dipeptidyl peptidase having an amino acid sequence comprising SEQ ID NO: 44;

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

4. Claim : 17 partially and 23 completely

A method of reducing growth of a bacterium comprising inhibiting a prolyl dipeptidyl peptidase, active analog, active fragment, or active modification thereof; a dipeptidyl peptidase having an amino acid sequence comprising SEQ ID NO: 45.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte nal Application No
PCT/US 00/05551

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 2005880 A	10-01-1990	NONE	

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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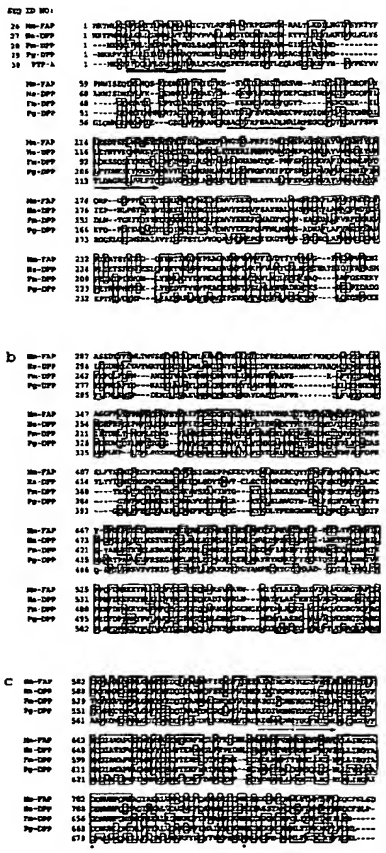
(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT,

[Continued on next page]

(54) Title: BACTERIAL PROLYL PEPTIDASES AND METHODS OF USE



(57) Abstract: The present invention provides isolated polypeptides, prolyl tripeptidyl-peptidases, and active analogs, active fragments or active modifications thereof, having amidolytic activity for cleavage of a peptide bond present in a target peptide having at least 30 amino acids. Isolated nucleic acid fragments encoding isolated prolyl tripeptidyl-peptidases are also provided, as are methods of reducing growth of a bacterium by inhibiting a prolyl tripeptidyl-peptidase.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

BACTERIAL PROLYL PEPTIDASES AND METHODS OF USE

CONTINUING APPLICATION DATA

This patent application claims the benefit of U.S. provisional patent application No. 60/123,148, filed March 5, 1999, which is incorporated by reference herein.

GOVERNMENT FUNDING

The present invention was made with government support under Grant No. DE 09761, awarded by the National Institutes of Health. The Government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

Porphyromonas gingivalis (formerly *Bacteroides gingivalis*) is an obligately anaerobic bacterium which is implicated in periodontal disease. *P. gingivalis* produces several distinct proteolytic enzymes, many of which are recognized as important virulence factors. A number of physiologically significant proteins, including collagen, fibronectin, immunoglobulins, complement factors C3, C4, C5, and B, lysozyme, iron-binding proteins, plasma proteinase inhibitors, fibrin and fibrinogen, and factors of the plasma coagulation cascade system, are hydrolyzed by *P. gingivalis* proteases. Broad proteolytic activity plays a role in the evasion of host defense mechanisms and the destruction of gingival connective tissue in progressive periodontitis.

Progressive periodontitis is characterized by acute tissue degradation promoted by collagen digestion and a vigorous inflammatory response characterized by excessive neutrophil infiltration. Gingival crevicular fluid accumulates in periodontitis as periodontal tissue erosion progresses at the foci of the infection, and numerous plasma proteins are exposed to proteinases expressed by the bacteria at the injury site. Neutrophils are recruited to the gingiva, in part, by the humoral chemotactic factor C5a. The complement components C3 and C5 are activated by complex plasma proteases with "trypsin-like" specificities called convertases. The human plasma convertases

cleave the α-chains of C3 and C5 at a specific site generating biologically active factors known as anaphylatoxins (i.e. C3a and C5a). The anaphylatoxins are potent proinflammatory factors exhibiting chemotactic and/or spasmogenic activities as well as promoting increased vascular permeability. The larger products from C3 and C5 cleavage (i.e. C3b and C5b) participate in functions including complement cascade activation, opsonization, and lytic complex formation.

Recent studies have indicated that this periodontopathogen produces at least seven different enzymes belonging to the cysteine and serine catalytic classes of peptidases, among which three cysteine proteinases (gingipains) are predominant (Potempa, J., et al. (1995) *Prospect. Drug Discovery and Design* 2, 445-458). The gingipains are the best characterized group of *P. gingivalis* enzymes as their structure, function, enzymatic properties and pathological significance are known. From *in vitro* studies it is apparent that two gingipains R (also referred to generally as "Arg-gingipains" and more specifically as RgpA and RgpB), enzymes specific for cleavage at Arg-Xaa peptide bonds, have a significant potential to contribute to the development and/or maintenance of a pathological inflammatory state in infected periodontal pockets through: (*i*) activation of the kallikrein-kinin cascade, (*ii*) the release of neutrophil chemotactic activity from native and oxidized C5 of the complement pathway, and (*iii*) activation of factor X. In addition, gingipain K (also referred to as "Lys-gingipain"), an enzyme which cleaves Lys-Xaa peptide bonds, degrades fibrinogen. This may add to a bleeding on probing tendency associated with periodontitis. Finally, the presence of a hemagglutinin/adhesion domain in the non covalent multiprotein complexes of RgpA and gingipain K suggests participation of these enzymes in the binding of *P. gingivalis* to extracellular matrix proteins which may facilitate tissue invasion by this pathogen.

In comparison to the gingipains, relatively little is known about other cysteine proteinases produced by *P. gingivalis*. Two genes, referred to as *tpr* and *prtT* have been cloned and sequenced and although they encode a putative papain-like and streptopain-like cysteine proteinases, respectively, neither has been purified and characterized.

The presence of serine proteinase activity in cultures of *P. gingivalis* has been known for several years; however, only limited information is available about such enzymes. Indeed, a serine endopeptidase has been isolated from culture media, although it was only superficially characterized (Hinode D., et al., (1993) *Infect. Immun.* 59, 3060-3068). On the other hand, an enzyme referred to as glycylprolyl peptidase (DPP IV) was found to be associated with bacterial surfaces and two molecular mass forms of this peptidase have been described. This enzyme has also been shown to possess the ability to hydrolyze partially degraded type I collagen, releasing the Gly-Pro dipeptide, and it was suggested that, in collaboration with collagenase, glycylprolyl peptidase may contribute to the destruction of the periodontal ligament (Abiko, Y., et al. (1985) *J.Dent. Res.* 64, 106-111). In addition to this potential pathological function, glycylprolyl peptidase may also play a vital role in providing *P. gingivalis* with dipeptides which can be transported inside the cell and serve as a source of carbon, nitrogen, and energy for this asaccharolytic organism. Recently, a gene encoding glycylprolyl peptidase in *P. gingivalis* has been cloned and sequenced, and it is now apparent that this enzyme is homologous to dipeptidyl-peptidase IV (DPP-IV) from other organisms (Kiyama, M., et al. (1998) 1396, 39-46). The nucleotide sequence of the genome of this bacterium is currently being determined by The Institute for Genomic Research, and is available at www.tigr.org.

SUMMARY OF THE INVENTION

The present invention is directed to an isolated prolyl tripeptidyl-peptidase, active analog, active fragment, or active modification thereof having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids. Alternatively, the isolated prolyl tripeptidyl-peptidase, active analog, active fragment, or active modification thereof is isolated from *P. gingivalis*. Typically, amidolytic activity is determined with a prolyl tripeptidyl-peptidase:target polypeptide ratio of at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours. The peptide cleaved by the isolated prolyl tripeptidyl-peptidase can include the sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID

NO:7, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:11, H-Ala-Arg-Pro-Ala-D-Lys-amide, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:25, or SEQ ID NO:37. The amino acid sequence of the isolated prolyl tripeptidyl-peptidase can include the amino acid sequence GXSXXG (SEQ ID NO:39), the amino acid sequence GXSXGG (SEQ ID NO:40), or the amino acid sequence of SEQ ID NO:30.

Another aspect of the invention is an isolated polypeptide, active analog, active fragment, or active modification thereof having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids. Typically, the polypeptide:target polypeptide ratio of at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours.

The invention is also directed to an isolated polypeptide comprising an amino acid sequence having a percentage amino acid identity of greater than 35 % with SEQ ID NO:30.

An alternative aspect of the invention is an isolated nucleic acid fragment encoding a prolyl-tripeptidyl peptidase, active analog, active fragment, or active modification thereof, having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids. Typically, the prolyl tripeptidyl-peptidase:target polypeptide ratio of at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours. The nucleic acid fragment can have a nucleotide sequence comprising SEQ ID NO:38. A complement of the nucleic acid fragment can hybridize to SEQ ID NO:38 under hybridization conditions of 0.5 M phosphate buffer, pH 7.2, 7 % SDS, 10 mM EDTA, at 68°C, followed by three for 20 minutes washes in 2x SSC, and 0.1 % SDS, at 65°C, wherein at least about 20 nucleotides of the complement hybridize.

Another aspect of the invention is an isolated nucleic acid fragment encoding a polypeptide that includes an amino acid sequence having a percentage amino acid identity of greater than 35 % with SEQ ID NO:30.

The invention is also directed at a method of identifying an inhibitor of a prolyl-tripeptidyl peptidase, active analog, active fragment, or active modification thereof, including identifying a molecule that inhibits the

amidolytic activity of the prolyl-tripeptidyl peptidase. The inhibitor is identified by incubating the prolyl-tripeptidyl peptidase with the molecule under conditions that promote amidolytic activity of the prolyl-tripeptidyl peptidase and determining if the amidolytic activity of the prolyl-tripeptidyl peptidase is inhibited relative to the amidolytic activity in the absence of molecule.

An aspect of the invention is a method of reducing growth of a bacterium. This method includes inhibiting a prolyl tripeptidyl-peptidase, active analog, active fragment, or active modification thereof, or a prolyl dipeptidyl-peptidase, active analog, active fragment, or active modification thereof. The method includes contacting the prolyl tripeptidyl-peptidase with an inhibitor of the prolyl tripeptidyl-peptidase. The method can be used to protect an animal from a periodontal disease caused by *P. gingivalis* including administering to the animal the inhibitor. The disease can be selected from the group consisting of gingivitis and periodontitis. The inhibitor can be administered by a method selected from the group consisting of subgingival application and controlled release delivery.

Another aspect of the invention is an immunogenic composition including an isolated prolyl tripeptidyl-peptidase, or an antigenic analog, antigenic fragment, or antigenic modification thereof, the prolyl tripeptidyl-peptidase having amidolytic activity for cleavage of a peptide bond present in a target peptide having at least 4 amino acids. Typically, the prolyl tripeptidyl-peptidase:target polypeptide ratio is at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours. The immunogenic composition can include an adjuvant.

The invention is also directed to a composition including an inhibitor of an isolated prolyl tripeptidyl-peptidase and a pharmaceutically acceptable carrier.

Additional aspects of the invention include a dipeptidyl peptidase having an amino acid sequence including SEQ ID NO:43, SEQ ID NO:44, or SEQ ID NO:45.

Definitions

"Polypeptide" as used herein refers to a polymer of amino acids and does not refer to a specific length of a polymer of amino acids. Thus, for example, the terms peptide, oligopeptide, protein, and enzyme are included within the definition of polypeptide. This term also includes post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations, and the like. A polypeptide can be produced by an organism, or produced using recombinant techniques, or chemically or enzymatically synthesized.

"Peptidase," "proteinase," and "protease" all refer to enzymes that catalyze the hydrolysis of peptide bonds in a polypeptide. A "peptide bond" or "amide bond" is a covalent bond between the alpha-amino group of one amino acid and the alpha-carboxyl group of another amino acid. "Peptidase inhibitor," "proteinase inhibitor," "protease inhibitor," and "inhibitor" all refer to molecules that inhibit a peptidase that catalyzes the hydrolysis of peptide bonds in a polypeptide.

As used herein, the term "isolated" means that a polypeptide or a nucleic acid fragment has been either removed from its natural environment, produced using recombinant techniques, or chemically or enzymatically synthesized. Preferably, the polypeptide or nucleic acid fragment is purified, i.e., essentially free from any other polypeptides or nucleic acid fragments and associated cellular products or other impurities.

"Amidolytic activity" refers to the ability of a polypeptide to catalyze the hydrolysis of at least one peptide bond in a polypeptide. The term "cleavage" can also be used to refer to the hydrolysis of a peptide bond in a polypeptide. "Prolyl-tripeptidyl peptidase" and "PTP" refer to a polypeptide having a particular "amidolytic activity". A "prolyl-tripeptidyl peptidase" is able to hydrolyze the peptide bond between the proline and the Yaa residues in a target polypeptide with the general formula NH_2-Xaa-Xaa-Pro-Yaa-$(Xaa)_n$ (SEQ ID NO:25), wherein Xaa is a natural or modified amino acid, Yaa is a natural or modified amino acid except proline, and the α-amino of the amino terminal residue is not blocked. A "prolyl tripeptidyl-peptidase" does not have to cleave all members of the target peptide. The term "natural amino acid" refers to the 20 amino acids typically produced by a cell. The term "modified amino acid"

refers to, for instance, acetylation, hydroxylation, methylation, amidation, and the attachment of carbohydrate or lipid moieties, cofactors, and the like.

A "target polypeptide" is a polypeptide that is the potential substrate of the amidolytic activity of a prolyl tripeptidyl-peptidase.

An active analog, active fragment, or active modification of a polypeptide of the invention is one that has amidolytic activity by hydrolysis of a peptide bond present in the target polypeptide as described herein. Active analogs, fragments, and modifications are described in greater detail herein.

"Nucleic acid fragment" as used herein refers to a linear polymeric form of nucleotides of any length, either ribonucleotides or deoxynucleotides, and includes both double- and single-stranded DNA and RNA. A nucleic acid fragment may include both coding and non-coding regions that can be obtained directly from a natural source (e.g., a microorganism), or can be prepared with the aid of recombinant or synthetic techniques. A nucleic acid molecule may be equivalent to this nucleic acid fragment or it can include this fragment in addition to one or more other nucleotides or polynucleotides, For example, the nucleic acid molecule of the invention can be a vector, such as an expression of cloning vector.

"Percentage amino acid identity" refers to a comparison of the amino acids of two polypeptides as described herein.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1. Purification of the prolyl tripeptidyl peptidase from the acetone precipitate of the *P. gingivalis* cell extracts. Absorbance at 280 nm (open triangles), amidolytic activity against H-Ala-Phe-Pro-pNA (closed diamonds), and H-Gly-Pro-pNA (closed circles). *(a)* Separation of PTP-A on hydroxyapatite. *(b)* Separation of PTP-A on Phenyl-Sepharose HP. *(c)* Separation of PTP-A on MonoQ FPLC. *(d)* Chromatofocusing of PTP-A on Mono-P.

Fig. 2. SDS -PAGE of fractions from purification of PTP-A and the autoradiography of the purified enzyme. *Lane a*, molecular mass markers (phosphorylase B, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa; α-lactalbumin, 14

kDa); *lane b*, acetone precipitate from Triton X-100 extract of *P. gingivalis*, *lane c*: hydroxyapatite column eluate; *lane d*, Phenyl-Sepharose column eluate; *lane e*, MonoQ column eluate; *lane f*, purified PTP-A from MonoP column wash; *lane g*, autoradiograph of ^{3}H-DFP labeled enzyme exposed for 96 h to X-ray film. All samples were reduced and boiled prior to PAGE analysis.

Fig. 3. Multiple sequence alignment of *P. gingivalis* PTP-A (PTP-A) and its bacterial and eukaryotic homologues. Pg-DPP, DPP from *P. gingivalis* (Kiyama, M., et al., (1998) *Bioch. Bioph. Acta* 1396, 39-46) containing an amino-terminal sequence corrected according to the *P. gingivalis* W83 genome data available from The Institute of Genomic Research at www.tigr.org); Fm-DPP, DPP from *Flavobacterium meningosepticum*; Hs-DPP, human DPP IV; and Mm-FAP, mouse fibroblast activation protein. Peptide sequences obtained from PTP-A analysis described herein are indicated with arrows (note that the sequence of the peptide 81-97 corresponds to the N-terminus of the lower molecular weight form of PTP-A); catalytic triad is marked with asterisks; and the proposed PTP-A membrane-anchoring N–terminal α-helix is double-underlined. Homologous regions (i.e., regions of identical amino acids and/or conservative substitutions) are highlighted. Identical regions are shown as white letters on a black background.

Fig. 4. Comparison of *P. gingivalis* PTP-A and DPP active site domains to corresponding sequences of three putative homologues identified within the *P. gingivalis* genome (DPP-H1, DPP-H2 and DPP-H3). Sequences of *P. gingivalis* PTP-A, DPP, DPP-H1, DPP-H2, and DPP-H3 were obtained from conceptual translation of the following open reading frames retrieved from The Institute for Genomic Research (TIGR) unfinished P. gingivalis genome database: gnl | TIGR | *P. gingivalis* contig 126 (positions 13 228 – 15 426), contig 87 (positions 6 424 – 4 399), contig 65 (positions 161 – 1 786), contig 101 (positions 8 895 – 6 845), and contig 9 (positions 4 216 – 2 162), respectively. Residues predicted as catalytic triads are marked with asterisks. Homologous regions (i.e., regions of identical amino acids and/or conservative substitutions) are highlighted.

Identical regions are shown as white letters on a black background. Similar regions (i.e., conservative substitutions) are shown as white letters on a grey background.

Fig. 5. Influence of Pefabloc-serine proteinase inhibitor on *P. gingivalis* growth.

Fig. 6. Comparison of *P. gingivalis* PTP-A and DPP to sequences of three putative homologues identified within the *P. gingivalis* genome (DPP-H1, DPP-H2 and DPP-H3). Sequences of *P. gingivalis* PTP-A (126PP), DPP (87PP), DPP-H1 (65PP), DPP-H2 (101PP), and DPP-H3 (9PP) were obtained as described in Fig. 4. Homologous regions (i.e., regions of identical amino acids and/or conservative substitutions) are highlighted. Identical regions are shown as white letters on a black background. Similar regions (i.e., conservative substitutions) are shown as white letters on a grey background.

Fig. 7. Nucleotide sequence (SEQ ID NO:38) and amino acid sequence (SEQ ID NO:30) of PTP-A.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides isolated polypeptides, preferably isolated prolyl peptidases, more preferably prolyl dipeptidyl-peptidases and prolyl tripeptidyl-peptidases, most preferably prolyl-tripeptidyl peptidases, that have amidolytic activity by hydrolysis of a peptide bond present in a target polypeptide, where the bond is between a proline and an amino acid residue attached to the alpha-carboxyl group end of the proline.

When the prolyl peptidase is a prolyl tripeptidyl-peptidase, the peptidase has amidolytic activity by hydrolysis of a peptide bond present in a target polypeptide of the general formula NH_2-Xaa-Xaa-Pro-Yaa-$(Xaa)_n$ (SEQ ID NO:25), wherein Xaa is a natural or modified amino acid, Yaa is a natural or modified amino acid except proline, and the α-amino of the amino terminal residue is not blocked, wherein the peptide bond of the target polypeptide that is hydrolyzed is the Pro-Yaa peptide bond. Preferably, isolated polypeptides do not cleave a target peptide having a blocked α-amino of the amino terminal residue.

Preferably, the only peptide bond of the target peptide that is hydrolyzed is the Pro-Yaa bond. In increasing order of preference, isolated polypeptides can cleave a target peptide that is at least 4 amino acids or at least 300 Da, at least 10 amino acids or at least 750 Da, at least 20 amino acids or at least 1,500 Da, or at least 30 amino acids or at least 3,000 Da. Preferably, the prolyl-tripeptidyl peptidases cleave peptides 1, 2, 7, 8, and 10-15 shown in Table 3, human cystatin C, and interleukin 6.

When the prolyl peptidase is a prolyl dipeptidyl-peptidase, the peptidase has amidolytic activity by hydrolysis of a peptide bond present in a target polypeptide of the general formula NH_2-Xaa-Zaa-Yaa-$(Xaa)_n$ (SEQ ID NO:12), wherein Xaa is a natural or modified amino acid, Zaa is a proline or alanine, Yaa is a natural or modified amino acid except proline or hydroxyproline, and the α-amino of the amino terminal residue is not blocked, wherein the peptide bond of the target polypeptide that is hydrolyzed is the Zaa-Yaa peptide bond. Preferably, isolated polypeptides does not cleave a target peptide having a blocked α-amino of the amino terminal residue. Preferably, the only peptide bond of the target peptide that is hydrolyzed is the Pro-Yaa bond.

Due to their cyclic aliphatic character proline residues bestow unique conformational constraints on polypeptide chain structures, significantly affecting the susceptibility of proximal peptide bonds to proteolytic cleavage. Those proline residues, which often appear near the amino-termini of many biologically active peptides, may protect them against proteolytic degradation by peptidases with general specificity. A specialized group of proteolytic enzymes, typically referred to as prolyl peptidases, has evolved to cleave (i.e., hydrolyze) a peptide bond adjacent to a proline residue in a polypeptide. The peptide bond adjacent to a proline residue can be referred to as a prolyl-X bond, where prolyl is the proline residue, and X is an amino acid residue attached to the alpha-carboxyl group end of the proline. The bacterial prolyl peptidases can cleave a polypeptide to liberate a tripeptide or a dipeptide. Prolyl peptidases that do not cleave a target peptide if the α-amino of the amino terminal residue is blocked can be referred to as exopeptidases. The *in vivo* activity of these specialized proteolytic enzymes may have important physiological significance, because it may lead to inactivation of many biologically active peptides and/or transformation of the activity of other

biologically active peptides. In addition, hydrolysis of prolyl-X bonds in conjunction with general catabolic pathways should allow the complete re-utilization of amino acids by living organisms, including bacteria. However, prolyl peptidases from bacterial pathogens, if released into the host environment, may interfere with the physiological functions of biologically active polypeptides and, therefore, contribute to the pathogenicity of infectious disease.

The external (i.e., cell surface) localization and uncontrolled activity of bacterial peptidases, including prolyl peptidases, likely contributes significantly to run-away inflammation in the human host and the pathological degradation of connective tissue during periodontitis. For instance, working in concert bacterial prolyl peptidases (e.g., prolyl tripeptidyl peptidases and DPP IV) have the ability to completely degrade collagen fragments locally generated by endogenous or bacterial collagenases. Because type I collagen is the major component of periodontal ligament, its enhanced degradation by bacterial prolyl peptidases may contribute to loss of tooth attachment and periodontal pocket formation. Thus, there is a need in the art to characterize bacterial peptidases to facilitate the development of therapies to inhibit the activity of the bacterial peptidases.

The polypeptides of the present invention, preferably prolyl peptidases, can be used as a source of antibodies for inhibiting the peptidase activity and thereby possibly reducing periodontitis, loss of tooth attachment and periodontal pocket formation. Antibodies to prolyl peptidases can also be used to identify and/or isolate additional prolyl peptidases. Knowledge of prolyl peptidases can also be used to make inhibitors of prolyl peptidases and to make immunogenic compositions that could be used to elicit the production of antibodies to prolyl peptidases and thereby possibly reduce gingivitis, periodontitis, loss of tooth attachment, and/or periodontal pocket formation.

An example of a prolyl-tripeptidyl peptidase is prolyl-tripeptidyl peptidase A (SEQ ID NO:30) (also referred to as PTP-A) from *P. gingivalis*. Purified PTP-A has apparent molecular masses of 81.8 and 75.8 kDa. The lower molecular mass peptidase may be due to the proteolytic cleavage of the peptidase from the surface of *P. gingivalis*. PTP-A is a new member of clan SC, family S9 of serine peptidases. Clans of serine peptidases are grouped on the basis of the order of certain amino acids in the polypeptide that make up the "catalytic triad" which

plays a pivotal role peptidase activity. The members of the clan SC are characterized by the catalytic triad in the polypeptide in the order of serine, aspartic acid, and histidine. Members of the clan SC are also characterized by a tertiary structure including β/α/β units, and an α/β hydrolase fold. In addition to the catalytic triad order, the amino acid sequence GXSXXG (SEQ ID NO:39), where X is any amino acid and S is the active site serine, is a signature of all members of the clan SC with some distinguishing features specific for each family. Family S9 has the consensus sequence GXSXGG (SEQ ID NO:40). Besides this consensus sequence, there is a general similarity of primary structures which classifies peptidases to this family. For instance, peptidases of this family generally have two domains, an amino-terminal domain that contains a membrane binding domain, and a carboxy-terminal domain, also referred to as the catalytic domain. The catalytic domain contains the residues of the catalytic triad. Some members of the S9 family have only the catalytic domain.

The S9 family is diverged and divided in three subfamilies: S9A, cytosolic oligopeptidases from archae and eukaryotes; S9B, eukaryotic acylaminoacylpeptidases; and S9C, dipeptidyl peptidease IV from bacteria and eukaryotes. The catalytic domain of peptidases from family S9 typically begin at about residue 400 of SEQ ID NO:30 and include the remaining carboxy-terminal amino acids (see, e.g., Fulop, et al., (1998) *Cell* 94, 161-170). Despite structural similarities to peptidases from the S9 family, the tripeptidyl-peptidase activity of PTP-A is unusual for this family of enzymes, and no other known similar activity has so far been attributed to any other member of the S9 family. In fact, all strict tripeptidyl-peptidases belong only to the subtilisin family (S8) and S33 family of serine peptidases; however, they neither share a structural relationship with PTP-A nor have activity limited to cleavage after proline residues. In particular, there are no other known prolyl tripeptidyl peptidases with an activity that is increased by iodoacetamide relative to the same prolyl tripeptidyl peptidase in the absence of iodoacetamide under the same conditions. Iodoacetamide is a compound that is traditionally a peptidase inhibitor. Typically, the activity of a prolyl tripeptidyl peptidase is increased about two-fold. Furthermore, unlike oligopepidases, the prolyl tripeptidyl-peptidases of the present invention can cleave target peptidases having as few as 4 amino acids but also target peptides having at least 30 amino

acids or a molecular weight of at least 3,000 Da. In these respects, the *P. gingivalis* tripeptidyl peptidase is a unique enzyme, and the isolation and characterization of this novel bacterial prolyl peptidase will facilitate the development of therapies to inhibit the activity of the bacterial peptidases.

Examples of putative prolyl-dipeptidyl peptidases are DPP-H1 (SEQ ID NO:43), DPP-H2 (SEQ ID NO:44), and DPP-H3 (SEQ ID NO:45). These peptidases have a significant percentage amino acid similarity with DPP IV and PTP-A (see Fig. 6). Each dipeptidyl peptidase is expected to have enzymatic activity, as each has a well preserved catalytic triad (Fig. 4). DPP IV has been characterized and the gene encoding the peptidase has been cloned, however the substrate specificity has not been well characterized. DPP IV has been found to cleave SEQ ID NOs:6, 20, 23, and 24. DPP IV has been purified in two forms. One of the forms is a full length gene translation product containing a blocked amino-terminal residue. The second form had the amino-terminal amino acid sequence HSYRAAVYDYDVRRNLVKPLSEHVG (SEQ ID NO:48), which corresponds to residues 116-140 of DPP IV (Kiyama, M., et al. (1998) 1396, 39-46), indicating that it was proteolytically truncated on the amino-terminus.

In *P. gingivalis*, PTP-A and DPP IV activity is cell surface associated. While not intending to be limiting, it is conceivable that the enzyme is membrane anchored through a putative signal sequence which is not cleaved but remains as a membrane spanning domain similar to other members of the prolyl oligopeptidase family. However, a significant portion of the purified PTP-A has a truncated N-terminus, apparently due to cleavage by Lys-specific peptidase and likely to be an artifact which has occurred during the purification procedure. Nevertheless, membrane bound PTP-A and DPP IV is proteolytically cleaved and shed during cultivation of the bacteria, as indicated by variable amount of soluble activities found in cell free culture media. The cell surface localization of PTP-A supports a putative physiological function in providing nutrients for growing bacterial cells. The inability of asaccharolytic *P. gingivalis* to utilize free amino acids makes the bacterium entirely dependant on an external peptide supply. In this regard, PTP-A and DPP IV activities are probably very important, if not indispensable, for bacterial growth, and inhibition of prolyl tripeptidyl-peptidases and dipeptidyl-peptidases may inhibit the *in vivo* growth of organisms, including *P. gingivalis*.

For instance, treatment of *P. gingivalis* cultures in lagphase (i.e., the period after inoculation of a culture and before the organism begins to divide) and early logarithmic growth with the inhibitors PEFABLOCK and 3,4-dichloroisocoumarin inhibits growth of *P. gingivalis*.

Preferably, a polypeptide of the invention, preferably a prolyl peptidase, contains the amino acid sequence GXSXXG (SEQ ID NO:39), most preferably, GXSXGG (SEQ ID NO:40), where G is glycine, X is any amino acid, and S is the active site serine. The active site serine can be identified by, for instance, labeling with diisopropylfluorophosphate as described herein. Preferably, the catalytic domain of the prolyl tripeptidyl-peptidases of the invention begins at about residue 400 of SEQ ID NO:30 and includes the remaining carboxy-terminal amino acids and the corresponding amino acids of SEQ ID NOs:43-45 (see Fig. 6), more preferably, at about residue 502 of SEQ ID NO:30 and includes the remaining carboxy-terminal amino acids and the corresponding amino acids of SEQ ID NOs:43-45 (see Fig. 6), most preferably, at about residue 556 of SEQ ID NO:30 and includes the remaining carboxy-terminal amino acids and the corresponding amino acids of SEQ ID NOs:43-45 (see Fig. 6).

The invention further includes a polypeptide, preferably a prolyl tripeptidyl-peptidase, that shares a significant level of primary structure with SEQ ID NO:30. The two amino acid sequences (i.e., the amino acid sequence of the polypeptide and the sequence SEQ ID NO:30) are aligned such that the residues that make up the catalytic triad, i.e., the serine, aspartic acid, and the histidine, are in register, then further aligned to maximize the number of amino acids that they have in common along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to place the residues of the catalytic triad in register and to maximize the number of shared amino acids, although the amino acids in each sequence must nonetheless remain in their proper order. The percentage amino acid identity is the higher of the following two numbers: (a) the number of amino acids that the two sequences have in common within the alignment, divided by the number of amino acids in SEQ ID NO:30, multiplied by 100; or (b) the number of amino acids that the two sequences have in common within the alignment, divided by the number of amino acids in the candidate polypeptide, multiplied by 100. Preferably, a prolyl tripeptidyl peptidase has

greater than 35 % identity, more preferably at least about 40 % identity, most preferably at least about 45 % identity with SEQ ID NO:30. Preferably, amino acids 154-732 of SEQ ID NO:30 are used, more preferably amino acids 400-732 of SEQ ID NO:30 are used. An isolated polypeptide comprising an amino acid sequence having a percentage amino acid identity of greater than 35 % with SEQ ID NO:30.

In general, the amidolytic activity of the polypeptides of the invention, preferably prolyl peptidases, can be measured by assay of the cleavage of a target polypeptide in the presence of prolyl peptidase and a buffer. Preferably, the lower ratio of prolyl tripeptidyl-peptidase to target polypeptide is at least about 1:1, more preferably at least about 1:100, even more preferably at least about 1:1,000, most preferably at least about 1:10,000. Preferably, the higher ratio of prolyl peptidase to target polypeptide is no greater than about 1:10,000,000, more preferably no greater than about 1:1,000,000 and most preferably no greater than about 1:100,000. Buffers in which a prolyl peptidase is active are suitable for the assay. Preferably, the buffer is about 200 mM HEPES (N-2-hydroxyethylpeperazine,N'-2-ethansulfonic acid), more preferably about 50 mM HEPES, most preferably about 20 mM HEPES. Preferably, the pH of the buffer is at least about pH 6.0 and no greater than pH 8.0, more preferably about pH 7.5. Preferably, the temperature of the assay is at about 37°C. The assay can be carried out for at least about 1 minute to no greater than 24 hours. Preferably, the amidolytic activity of the prolyl peptidases are measured at a prolyl peptidase:target polypeptide ratio of at least about 1:100 to no greater than 1:1,000,000 in about 200 mM HEPES, about pH 7.5 at about 37°C for at least about 3 hours. In general, the time of the assay can vary depending on the substrate and enzyme:substrate ratio. Typically, target peptides are stable under these conditions, and typically it is difficult to detect background levels of hydrolysis in the absence of a prolyl peptidase. Preferably, the assay is allowed to continue until at least 1 % of the target peptide is hydrolyzed.

Prolyl-tripeptidyl peptidases of the present invention preferably are inhibited by a compound chosen from the group consisting of PEFABLOCK (4-(2-aminoethyl)-benzenesulfonyl-fluoride hydrochloride), diisopropylfluorophosphate, and 3,4-dichloroisocoumarin, more preferably PEFABLOCK and diisopropylfluorophosphate, and most preferably diisopropylfluorophosphate. The

peptidases of the present invention are preferably not inhibited by a compound chosen from the group consisting of leupeptin, antipain, E-64, pepstatin, α_1-proteinase inhibitor, α_1–antichymotrypsin and α_2–macroglobulin, most preferably. Significantly and unexpectedly, the amidolytic activity of a prolyl-tripeptidyl peptidase of the present invention is increased by iodoacetamide relative to the prolyl-tripeptidyl peptidase in the absence of iodoacetamide under the same conditions. Preferably, the effect of iodoacetamide on amidolytic activity is measured by incubating in 200 mM HEPES, pH 7.6, at least about 0.1 nM of the prolyl tripeptidyl-peptidase with the inhibitor for about 15 minutes, adding about 1 mM of H-Ala-Phe-Pro-pHA, and incubating for at least about 1 minute before assaying for amidolytic activity. Typically, at least about 1 mM to no greater than 100 mM of inhibitor is used.

The polypeptides of the invention include a polypeptide having SEQ ID NO:30, or an active analog, active fragment, or active modification of SEQ ID NO:30. An active analog, active fragment, or active modification of a polypeptide having SEQ ID NO:30 is one that has amidolytic activity by hydrolysis of the Pro-Yaa peptide bond present in a target polypeptide of the general formula NH_2-Xaa-Xaa-Pro-Yaa-$(Xaa)_n$ (SEQ ID NO:25). Active analogs of a polypeptide having SEQ ID NO:30 include prolyl-tripeptidyl peptidases having amino acid substitutions that do not eliminate hydrolysis of SEQ ID NO:25 at the Pro-Yaa peptide bond. Substitutes for an amino acid may be selected from other members of the class to which the amino acid belongs. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and tyrosine. Polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Examples of preferred conservative substitutions include Lys for Arg and *vice versa* to maintain a positive charge; Glu for Asp and *vice versa* to maintain a negative charge; Ser for Thr so that a free -OH is maintained; and Gln for Asn to maintain a free NH_2.

Active fragments of a prolyl-tripeptidyl peptidase of the invention include prolyl-tripeptidyl peptidases containing deletions or additions of one or more contiguous or noncontiguous amino acids such that the resulting polypeptide will

hydrolyze SEQ ID NO:25 at the Pro-Yaa peptide bond. An example of a fragment of a prolyl-tripeptidyl peptidase is a catalytic domain. Modified prolyl-tripeptidyl peptidases include prolyl-tripeptidyl peptidases that are chemically and enzymatically derivatized at one or more constituent amino acid, including side chain modifications, backbone modifications, and N- and C- terminal modifications including acetylation, hydroxylation, methylation, amidation, and the attachment of carbohydrate or lipid moieties, cofactors, and the like. Modified prolyl-tripeptidyl peptidases will hydrolyze SEQ ID NO:25 at the Pro-Yaa peptide bond.

Prolyl peptidases can be obtained by several methods. Isolation of a prolyl-tripeptidyl peptidase present on the surface of a cell producing the peptidase typically requires lysis of the cell followed by purification methods that are well known in the art. Alternatively, cels can be treated with a detergent, for instance Triton X-100, to remove the peptidase from the cell surface. The following are nonlimiting examples of suitable protein purification procedures: fractionation on immunoaffinity, ion-exchange, hydroxyapatite, Phenyl-Sepharose HP, MonoQ HR 5/5, or MonoP columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on an ion-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75. Preferably, isolation of a prolyl-tripeptidyl peptidase from *P. gingivalis* is accomplished using a combination of hydroxyapatite, Phenyl-Sepharose HP, MonoQ HR 5/5 and MonoP column chromatography steps as described herein.

Prolyl peptidases can also be isolated from organisms other than *P. gingivalis*. Other organisms can express a prolyl-tripeptidyl peptidase that is encoded by a coding region having similarity to the PTP-A coding region. A "coding region" is a linear form of nucleotides that encodes a polypeptide, usually via mRNA, when placed under the control of appropriate regulatory sequences. The boundaries of a coding region are generally determined by a translation start codon at its 5' end and a translation stop codon at its 3' end. "Regulatory region" refers to a nucleic acid fragment that regulates expression of a coding region to which a regulatory region is operably linked. Non limiting examples of regulatory regions include promoters, transcription initiation sites, translation start sites, translation

stop sites, and terminators. "Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A regulatory element is "operably linked" to a coding region when it is joined in such a way that expression of the coding region is achieved under conditions compatible with the regulatory region. Alternatively, other organisms can express a prolyl-tripeptidyl peptidase from a recombinant coding region encoding the peptidase. The identification of similar coding regions in other organisms can be accomplished as described herein. A prolyl-tripeptidyl peptidase can be isolated using purification methods that are well known in the art. Alternatively, the peptidase can be chemically synthesized using methods that are well known in the art including, for instance, solid phase synthesis. Examples of, for instance, coding and regulatory regions are described herein.

The expression of a prolyl-tripeptidyl peptidase by an organism other than *P. gingivalis* can be detected using specific substrates of the general formula NH_2-Xaa-Xaa-Pro-LG or NH_2-Xaa-Xaa-Pro-Yaa (SEQ ID NO:41), where LG is a leaving group. The leaving group can be a chromogenic or fluorogenic group known to the art. The expression of a prolyl-tripeptidyl peptidase by an organism and subsequent cleavage of a specific substrate results in a free amino acid or a free leaving group, each of which can be assayed using techniques known to those of skill in the art. Other methods can be based on immunogenic properties of PTP-A, for instance immunoassays and histochemistry, the detection of mRNA, and PCR related methods, all of which are known to one of skill in the art.

As described in the Examples, the amino acid sequence of the amino-terminal end of a PTP-A fragment was used to identify the nucleotide sequence of the PTP coding region. The nucleotide sequence was present in a publically available database containing the nucleotide sequence of the partially finished *P. gingivalis* W83 genome. However, even though the nucleotides that encode the *P. gingivalis* PTP-A were known, there was no indication that the nucleotides were in fact transcribed and translated. The data obtained from the database only contained the nucleotide sequence of a genomic clone; there was no disclosure that the nucleotides did or did not contain an open reading frame. Moreover, there is little data known to the art regarding regulatory regions required for either the transcription or the translation of a nucleotide sequence in *P. gingivalis*.

Thus, a person of ordinary skill, having the nucleotide sequence of the genomic clone, would not be able to predict that the open reading frame encoding PTP-A was transcribed or translated. Moreover, even if there was a suggestion that the open reading frame was both transcribed and translated, there is no suggestion that the polypeptide encoded by the open reading frame would have the novel activity of PTP-A.

Accordingly, the present invention is directed to a nucleic acid fragment encoding a polypeptide, particularly a prolyl-tripeptidyl peptidase, active analog, active fragment, or active modification thereof. The nucleic acid fragment can have a nucleotide sequence as shown in SEQ ID NO:38. Alternatively, nucleic acid fragments of the invention include those whose complement hybridize to SEQ ID NO:38 under standard hybridization conditions as described herein. During hybridization the entire nucleotide sequence of the complement can hybridize with SEQ ID NO:38. Preferably, at least about 20 nucleotides of the complement hybridize with SEQ ID NO:38, more preferably at least about 50 nucleotides, most preferably at least about 100 nucleotides.

Alternatively, the nucleic acid fragment can have a nucleotide sequence encoding a polypeptide having the amino acid sequence shown in SEQ ID NO:30. An example of the class of nucleotide sequences encoding such a polypeptide is SEQ ID NO:38. This class of nucleotide sequences is large but finite, and the nucleotide sequence of each member of the class can be readily determined by one skilled in the art by reference to the standard genetic code.

The identification of similar coding regions in other organisms can be accomplished by screening individual wild-type microorganisms for the presence of nucleotide sequences that are similar to the coding region of PTP-A, which is shown in SEQ ID NO:38. Screening methods include, for instance, hybridization of a detectably labeled probe with a nucleic acid fragment.

Standard hybridizing conditions are a modification of the conditions used by Church et al. ((1984) *Proc. Natl. Acad. Sci. USA* 81, 1991): 0.5 M phosphate buffer, pH 7.2, 7 % SDS, 10 mM EDTA, at 68°C, and three washes, each for 20 minutes in 2x SSC, 0.1 % SDS, at 65°C. Preferably, a probe will hybridize to the nucleotide sequence set forth in SEQ ID NO:38 under standard hybridizing conditions. Generally the probe does not have to be complementary to all the

nucleotides of the nucleic acid fragment as long as there is hybridization under the above-stated conditions.

"Complement" and "complementary" refer to the ability of two single stranded nucleic acid fragments to base pair with each other, where an adenine on one nucleic acid fragment will base pair to a thymine on a second nucleic acid fragment and a cytosine on one nucleic acid fragment will base pair to a guanine on a second nucleic acid fragment. Two nucleic acid fragments are complementary to each other when a nucleotide sequence in one nucleic acid fragment can base pair with a nucleotide sequence in a second nucleic acid fragment. For instance, 5'-ATGC and 5'-GCAT are complementary. The term complement and complementary also encompasses two nucleic acid fragments where one nucleic acid fragment contains at least one nucleotide that will not base pair to at least one nucleotide present on a second nucleic acid fragment. For instance the third nucleotide of each of the two nucleic acid fragments 5'-ATTGC and 5'-GCTAT will not base pair, but these two nucleic acid fragments are complementary as defined herein. Typically two nucleic acid fragments are complementary if they hybridize under the standard conditions referred to herein.

Preferred probes are nucleic acid fragments complementary to a coding region or another nucleotide sequence that encodes a prolyl-tripeptidyl peptidase. For instance, a probe can comprise a consecutive series of nucleotides complementary to a portion of SEQ ID NO:38. Preferably a probe is at least about 18 bases, more preferably at least about 21 bases, and most preferably at least about 24 bases in length. Particularly preferred probes are TTCGATCCGGCAAAGAAATATCCTGTTATTGTCTATGTTTACGGAGGAC CT (SEQ ID NO:36, GTGGATGCCGATAGAATAGGAGTACATGGCTGGAGCTATGGTGGCTTT (SEQ ID NO:37, and SEQ ID NO:38. Methods of detectably labeling a probe are well known to the art.

The nucleic acid fragment that is identified by the probe is further analyzed to determine if it encodes a polypeptide with amidolytic activity of the Pro-Yaa

peptide bond on a target polypeptide of the general formula NH_2-Xaa-Xaa-Pro-Yaa-$(Xaa)_n$ (SEQ ID NO:25). Another method for screening individual microorganisms for the presence of nucleotide sequences that are similar to the coding regions of the present invention is the polymerase chain reaction (PCR).

Individual wild-type microorganisms containing nucleic acid fragments encoding a prolyl-tripeptidyl peptidase can also be identified using antibody. Preferably the antibody is directed to PTP-A. The production of antibodies to a particular polypeptide is known to a person of skill in the art, and is further detailed herein.

The use of hybridization of a probe to a coding region present in individual wild-type microorganisms can be used as a method to identify a coding region identical or similar to a coding region present in SEQ ID NO:38. The coding region can then be isolated and ligated into a vector as described below. Two nucleic acid sequences are "similar" if the two nucleic acid sequences can be aligned so that the number of identical amino acids along the lengths of their sequences are optimized. Preferably, two nucleotide acid sequences have, in increasing order of preference, preferably at least about 90 %, at least about 92 %, at least about 94%, at least about 96%, most preferably at least about 98% identity.

As mentioned above, a nucleic acid fragment of the invention can be inserted in a vector. Construction of vectors containing a nucleic acid fragment of the invention employs standard ligation techniques known in the art. See, e.g., Sambrook et al, *Molecular Cloning: A Laboratory Manual.*, Cold Spring Harbor Laboratory Press (1989) or Ausubel, R.M., ed. *Current Protocols in Molecular Biology* (1994). A vector can provide for further cloning (amplification of the nucleic acid fragment), i.e., a cloning vector, or for expression of the polypeptide encoded by the coding region, i.e., an expression vector. The term vector includes, but is not limited to, plasmid vectors, viral vectors, cosmid vectors, or artificial chromosome vectors. Typically, a vector is capable of replication in a bacterial host, for instance *E. coli*. Preferably the vector is a plasmid.

Selection of a vector depends upon a variety of desired characteristics in the resulting construct, such as a selection marker, vector replication rate, and the like. Suitable plasmids for expression in *E. coli*, for example, include pUC(X), pKK223-3, pKK233-2, pTrc99A, and pET-(X) wherein (X) denotes a vector family in which

numerous constructs are available. pUC(X) vectors can be obtained from Pharmacia Biotech (Piscataway, NH) or Sigma Chemical Co. (St. Louis, MO). pKK223-3, pKK233-2 and pTrc99A can be obtained from Pharmacia Biotech. pET-(X) vectors can be obtained from Promega (Madison, WI) Stratagene (La Jolla, CA) and Novagen (Madison, WI). To facilitate replication inside a host cell, the vector preferably includes an origin of replication (known as an "ori") or replicon. For example, ColE1 and P15A replicons are commonly used in plasmids that are to be propagated in *E. coli*.

An expression vector optionally includes regulatory regions operably linked to the coding region. The invention is not limited by the use of any particular promoter, and a wide variety are known. Promoters act as regulatory signals that bind RNA polymerase in a cell to initiate transcription of a downstream (3' direction) coding region. The promoter used in the invention can be a constitutive or an inducible promoter. It can be, but need not be, heterologous with respect to the host cell. Preferred promoters for bacterial transformation include *lac*, *lac*UV5, *tac*, *trc*, T7, SP6 and *ara*.

An expression vector can optionally include a Shine Dalgarno site (e.g., a ribosome binding site), and a start site (e.g., the codon ATG) to initiate translation of the transcribed message to produce the enzyme. It can also include a termination sequence to end translation. A termination sequence is typically a codon for which there exists no corresponding aminoacetyl-tRNA, thus ending polypeptide synthesis. The nucleic acid fragment used to transform the host cell can optionally further include a transcription termination sequence. The rrnB terminators, which is a stretch of DNA that contains two terminators, T1 and T2, is an often used terminator that is incorporated into bacterial expression systems (J. Brosius et al., (1981) *J. Mol. Biol.* 148 107-127).

The nucleic acid fragment used to transform the host cell optionally includes one or more marker sequences, which typically encode a polypeptide that inactivates or otherwise detects or is detected by a compound in the growth medium. For example, the inclusion of a marker sequence can render the transformed cell resistant to an antibiotic, or it can confer compound-specific metabolism on the transformed cell. Examples of a marker sequence are sequences that confer resistance to kanamycin, ampicillin, chloramphenicol, and tetracycline.

Antibodies can be produced to a polypeptide having the sequence of SEQ ID NOs:30, 43, 44 or 45, or a polypeptide having a percentage amino acid identity as described herein. Alternatively, antibodies can be made to an antigenic analog, antigenic fragment, or antigenic modification of a polypeptide having the sequence of SEQ ID NOs:30, 43, 44 or 45. An antigenic analog, antigenic fragment, or antigenic modification of a polypeptide having SEQ ID NOs:30, 43, 44 or 45 is one that generates an immune response in an animal. Antigenic analogs of a polypeptide having SEQ ID NOs:30, 43, 44 or 45 include prolyl peptidases having amino acid substitutions that do not eliminate peptide antigenicity in an animal. Substitutes for an amino acid may be selected from other members of the class to which the amino acid belongs, as described herein. Fragments of a prolyl peptidase of the invention include prolyl peptidases containing deletions or additions of one or more contiguous or noncontiguous amino acids such that the resulting polypeptide will generate an immune response in an animal. An example of a fragments of a prolylpeptidase is a catalytic domain. Modified prolyl peptidases include prolyl peptidases that are chemically and enzymatically derivatized at one or more constituent amino acids, including side chain modifications, backbone modifications, and N- and C- terminal modifications including acetylation, hydroxylation, methylation, amidation, and the attachment of carbohydrate or lipid moieties, cofactors, and the like.

Accordingly, an aspect of the invention is an immunogenic composition comprising an isolated prolyl peptidase, or an antigenic analog, antigenic fragment, or antigenic modification thereof, preferably a prolyl tripeptidyl-peptidase. The prolyl tripeptidyl-peptidase preferably has amidolytic activity for cleavage of the Pro-Yaa peptide bond present in a target polypeptide with the general formula NH_2-Xaa-Xaa-Pro-Yaa-$(Xaa)_n$ (SEQ ID NO:25), wherein the amidolytic activity is measured at a prolyl tripeptidyl-peptidase:target polypeptide ratio of at least about 1:100 to no greater than about 1:1,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours.

The immunogenic composition can further include excipients or diluents that are pharmaceutically acceptable as carriers and compatible with the immunogenic composition. The term "pharmaceutically acceptable carrier" refers to a carrier(s) that is "acceptable" in the sense of being compatible with the other

ingredients of a composition and not deleterious to the recipient thereof. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the immunogenic composition may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the immune-stimulating composition.

The immunogenic composition can be used in a method for protecting an animal from a disease caused by *P. gingivalis*. This method includes administering the immunogenic composition and eliciting antibodies to a prolyl peptidase, antigenic analog, antigenic fragment, or antigenic modification. The diseases that can be treated in this manner include periodontal diseases, which includes gingivitis and periodontitis. Clinical hallmarks of periodontitis include lose of tooth attachment and periodontal pocket formation.

Alternatively and preferably, periodontal diseases can be treated by the use of inhibitors of a prolyl peptidase. An inhibitor of a prolyl peptidase, preferably a prolyl tripeptidyl-peptidase, can be present in a composition that preferably contains a pharmaceutically acceptable carrier. For instance, inhibitors can be applied systemically, subgingivally (e.g., subgingival irrigation) and/or by controlled release delivery directly into the periodontal pocket using methods well known to the art (see, e.g., Kornman, K., (1993) *J. Periodontol.* 64, 782-791). Preferably, an inhibitor is applied subgingivally or by controlled release delivery.

The prolyl peptidases, active analogs, fragments, and modifications thereof can be used in a method of reducing growth of bacteria *in vitro* or *in vivo*. Preferably, the bacteria is a periodontal pathogen, i.e, a bacterial pathogen that causes periodontal disease, more preferably the bacteria is *P. gingivalis*. The inability of asaccharolytic *P. gingivalis* to utilize free amino acids makes the bacterium entirely dependant on an external peptide supply. The action of the polypeptides of the invention may be required for bacterial growth, and inhibition of the polypeptides of the invention may inhibit the *in vivo* growth of organisms, including *P. gingivalis*. The method includes decreasing the amount of dipeptides and/or tripeptides (e.g., the result of cleavage of SEQ ID NO:25 by a prolyl-tripeptidyl peptidase) and the amount of free amino acids that result from further cleavage of the dipeptides and/or tripeptides present by inhibiting a prolyl

peptidase, active analog, active fragment, or active modification thereof, such that the amount of dipeptides and/or tripeptides generated by the polypeptides is decreased. The amount of dipeptides and/or tripeptides is decreased relative to the amount of dipeptides and/or tripeptides present in the absence of the inhibitor. Preferably, the amount of dipeptides and/or tripeptides generated is decreased by an inhibitor, a monoclonal antibody that inhibits the prolyl peptidase, or polyclonal antibodies that inhibit the prolyl peptidase, more preferably, the amount of dipeptides and/or tripeptides generated is decreased by an inhibitor. Preferably, an inhibitor acts to inhibit a polypeptide of the invention, preferably a prolyl peptidase, by blocking the active site of the polypeptide. The polypeptide can be present on the surface of the bacteria or secreted into the environment, preferably the polypeptide is present in the surface of the bacteria.

The present invention is also directed to a method of developing an inhibitor of a prolyl peptidase, active analog, active fragment, or active modification thereof, preferably a prolyl-tripeptidyl peptidase. The method includes identifying a molecule that inhibits the amidolytic activity of the prolyl peptidase. This can be accomplished by, for instance, incubating the prolyl peptidase with a candidate molecule under conditions that promote amidolytic activity of the prolyl peptidase and determining if the amidolytic activity of the prolyl peptidase is decreased relative to the amidolytic activity in the absence of the molecule. The amidolytic activity can be measured by cleavage of the Pro-Yaa peptide bond present in the target polypeptide SEQ ID NO:25 as described herein. One method of developing an inhibitor includes using the target peptide SEQ ID NO:25 and replacing the Xaa residues with modified amino acids. It is expected that some modified amino acids will cause the target peptide to act as an inhibitor.

EXAMPLES

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

Example 1

Materials

Diisopropylfluorophosphate (DFP), leupeptin and 3,4-dichloroisocoumarin, were purchased from Calbiochem (La Jolla, CA). Antipain, iodoacetamide, substance P, bradykinin and bradykinin related peptides, were obtained from Sigma. Other peptides used in this study were synthesized at the Molecular Genetic Instrumental Facility (University of Georgia, Athens, GA) using Fmoc protocol with an advanced ChemTech MPS350 automated synthesizer. H-Ala-Phe-Pro-pNA, H-Gly-Pro-pNA, Z-Gly-Pro-pNA, Z-Ala-Pro-pNA, and H-Pro-pNA (where pNA is p- Nitroanilide; Z is benzyloxycarbonyl; and H is hydrogen and denotes an unblocked amino-terminal group) were obtained from Bachem (King of Prussia, PA). Prolinal was kindly provided by Dr. James Powers (Georgia Institute of Technology, Atlanta) and cystatin C by Dr. Magnus Abrahamson (University of Lund, Sweden).

Methods

Source and Cultivation of Bacteria— P. gingivalis HG66 was obtained from Dr. Roland Arnold (University of North Carolina, Chapel Hill), while the strains W50 (ATCC 53978) and ATCC 33277 were obtained from the ATCC. All cells were grown as described previously (Chen, Z., et al., (1992) *J. Biol. Chem.* 267, 18896-18901).

Enzyme Activity Assays— Routinely, the tripeptidyl peptidase amidolytic activity was measured with H-Ala-Phe-Pro-pNA (1mM) in 0.2 M HEPES (N-2-hydroxyethylpeperazine,N'-2-ethansulfonic acid), pH 7.5 at 37°C. The concentration of enzyme was 0.1 nM to 1 nM. The assay was performed in a total volume of 0.2 ml on microplates, and the initial turnover rate was recorded at 405 nm using a microplate reader (Spectramax Molecular Devices, Sunnyvale, CA). In inhibition studies, the enzyme was first preincubated with inhibitor for 15 min at 37°C, substrate added, and residual activity recorded after 5 minutes to 30 minutes. H-Gly-Pro-pNA, Z-Ala-Pro-pNA, Z-Gly-Pro-pNA and H-Pro-pNA (1 mM final concentration) were assayed in the same manner.

Protein Determination—Protein concentration was determined using the BCA reagent kit (Sigma, St. Louis, MO), using bovine serum albumin as a standard.

Localization of Tripeptidyl-Peptidase Activity—Cultures of *P. gingivalis* HG66, W50 and ATCC 33277, at different phases of growth, were subjected to the following fractionation procedure. The cells were removed by centrifugation (10,000 x g, 30 minutes), washed twice with 10 mM Tris, 150 mM NaCl, pH 7.4, resuspended in 50 mM Tris, pH 7.6, and disintegrated by ultrasonication in an ice bath at 1500 Hz for 5 cycles (5 minutes sonication/5 minutes brake). Unbroken cells and large debris were removed by centrifugation (10,000 x g, 30 minutes) and the opalescent supernatant subjected to ultracentrifugation (150,000 x g, 120 minutes), yielding a pellet containing bacterial membranes and a supernatant which was considered as membrane-free cell extract. All fractions, as well as the full culture, culture medium, and full culture after sonication, were assayed for amidolytic activity against H-Ala-Phe-Pro-pNA.

Enzyme Purification—All purification steps were performed at 4°C except for FPLC separations, which were carried out at room temperature. Cells were harvested by centrifugation (6,000 x g, 30 minutes), washed with 50 mM potassium phosphate buffer, pH 7.4, and resuspended in the same buffer (150 ml per 50 gram of cells wet weight). Triton X-100 (10% volume/volume in H_2O) was added slowly to the bacterial cell suspension to a final concentration of 0.05%. After 120 minutes of gentle stirring, unbroken cells were removed by centrifugation (28,000 x g, 60 minutes). Proteins in the supernatant were precipitated with cold acetone (-20°C) added to a final concentration of 60% and collected by centrifugation. The pellet was redissolved in 50 mM potassium phosphate buffer, pH 7.0, and extensively dialyzed against 20 mM potassium phosphate, pH 7.0, containing 0.02% sodium azide. The dialyzed fraction was clarified by centrifugation (28,000 x g, 30 min) and applied to a hydroxyapatite column (BioRad, Melville, NY) equilibrated with 20 mM potassium phosphate, pH 7.0, at a flow rate of 20 ml/hour. After equilibration, the column was washed until the A_{280} fell to zero. Bound proteins were eluted with a gradient from 20-300 mM potassium phosphate and fractions (7 ml) analyzed for dipeptidyl- and tripeptidyl-peptidase activity using H-Gly-Pro-pNA and H-Ala-Phe-Pro-pNA, respectively. The activity against the latter

substrate was pooled, saturated with 1 M ammonium sulfate, clarified by centrifugation, and directly loaded onto a Phenyl-Sepharose HP (Pharmacia, Piscataway, NJ) column equilibrated with 50 mM potassium phosphate, pH 7.0, containing 1 M ammonium sulfate. The column was washed with two volumes of equilibration buffer, followed by buffer containing 0.5 M ammonium sulfate, and developed with a descending gradient of ammonium sulfate from 0.5 to 0 M. Active fractions were pooled, extensively dialyzed against 20 mM Tris, pH 7.5, and applied to a MonoQ HR 5/5 FPLC column equilibrated with the same buffer. The column was washed with 5 volumes of equilibration buffer at 1.0 ml/minute, following which bound proteins were eluted with a gradient of 0- 300 mM NaCl. The active fractions were pooled, dialyzed against 25 mM Bis-Tris, pH 6.3, and subjected to chromatofocusing on a MonoP FPLC column equilibrated with Bis-Tris buffer, using a pH gradient developed with 50 ml of 10x diluted Polybuffer 74 (Pharmacia), adjusted to a pH of 4.0.

Electrophoretic Techniques— The SDS-PAGE system of Schagger and von Jagow (Schagger, H., and von Jagow, G. (1987) *Anal. Biochem.* 166, 368-379), was used to monitor enzyme purification and estimate the enzyme molecular mass. For amino-terminal sequence analysis, proteins resolved in SDS-PAGE were electroblotted to polyvinylidene difluoride membranes using 10 mM CAPS, pH 11, 10% methanol (Matsudaira, P. (1987) *J. Biol. Chem.* 262, 10035-10038). The membrane was washed thoroughly with water and stained with Coomasie Blue G250. The blot was air dried, and protein bands cut out and subjected to NH_2-terminal sequence analysis with an Applied Biosystems 491 Protein Sequencer using the program designed by the manufacturers.

Enzyme Fragmentation —The purified prolyl tripeptidyl peptidase (PTP-A) was partially denatured by incubation in 6 M urea in 0.02 M Tris, pH 7.6, for 60 minutes. Low molecular mass gingipain R (RgpB) (Potempa, J., et al. (1995) *Prospect. Drug Discovery and Design* 2, 445-458) from *P. gingivalis* was then added to make an enzyme:substrate molar ratio of 1:100. The reaction mixture was made in 1 mM cysteine and the sample incubated overnight at 37°C. Generated peptides were separated by reverse-phase HPLC using a μBondapak C-18 column (3.9 x 300 mm) (Waters, Millford, MA). Peptides were eluted with 0.1% trifluoroacetic acid and acetonitrile containing 0.08% trifluoroacetic acid, using a

gradient from 0 to 80% acetonitrile over 60 minutes. Peptides were monitored at 220 nm and collected manually.

For determination of the active site serine residue and to confirm that the purified enzyme was a serine peptidase, 100 μg of purified PTP-A was first incubated with 170 μCi of [1,3-^{3}H]DFP (Amersham, Arlington Heights, IL) for 30 minutes at 25°C in 20 mM HEPES, pH 7.5. The reaction was quenched by addition of cold DFP to a final concentration of 10 mM and the radiolabelled material analyzed by SDS-PAGE, followed by autoradiographic analysis. The gel was dehydrated, soaked in PPO solution for 2 hours, dried, and the DFP-binding proteins detected by fluorography after an exposure time of 96 hours on X-ray film (XAR; Kodak, Rochester, NY). The bulk of radiolabelled protein was subjected to proteolytic fragmentation with RgpB and peptides obtained separated by reverse-phase HPLC as described above. Radioactivity in each peptide fraction was measured using a β liquid scintillation counter, and the labeled peptide, as well as other selected peptides were subjected to sequence analysis.

Identification of the PTP-A Gene— The database containing the unfinished *P. gingivalis* W83 genome, available from The Institute for Genomic Research, was searched for the presence of nucleotide sequences corresponding to the NH_2-terminal and the internal PTP-A amino acid sequences using the TBLASTN algorithm, BLAST version 2.0.8, and the default values for all parameters (Altschul, S.F., et al.,(1997) *Nucleic Acid Res.* 25, 3389-3402). An identified clone gnl | TIGR | P. gingivalis_126 was retrieved from The Institute for Genomic Research data base (http://www.tigr.org). The position of the PTP-A gene was localized using the NCBI open reading frame (ORF) finder (available from the National Center for Biotechnology Information, at http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The amino acid sequence, obtained by conceptual translation of the entire ORF, was further used for homology screening by use of the NCBI BLAST search tool.

Enzyme Specificity— Peptides were incubated with 1 μg PTP-A at an enzyme:substrate molar ratio of 1:100 for 3 hours or 24 hours in 50 μl of 200 mM HEPES, pH 7.5, at 37°C, and the reaction stopped by acidification with trifluoroacetic acid. The samples were then subjected to reverse-phase high

pressure liquid chromatography using a μBondapak C-18 column (3.9 x 300 mm) (Waters, Millford, MA) and an acetonitrile gradient (0-80 % in 0.075% trifluoroacetic acid in 50 min). Each peak, detected at 220 nm, was collected, lyophilized, re-dissolved in 50% (volume/volume) methanol, 0.1% acetic acid and subjected to analysis by mass spectrometry.

Mass Spectrometry—A Finnigan MAT 95S, sector mass spectrometer (Finnigan MAT, Bremen, Germany) equipped with an electrospray source (ESI) was used operated essentially as described previously (Stenfors, C., et al., (1997) *J. Biol. Chem.* 272, 5747-5751). Peptides were identified by fitting of the obtained spectra to specific sequences using an Internet application program MsFit available at http://falcon.ludwig.ucl.ac.uk/msfit.html.

Example 2

Enzyme Localization, Purification and Initial Characterization

Analysis of amidolytic activity against H-Ala-Phe-Pro-pNA in several fractions of *P. gingivalis* HG66, W50 and ATCC 33277 clearly indicated that an enzyme(s) with prolyl tripeptidyl-peptidase activity is localized on the cell surface in all strains tested with less than 5% of the total activity being found in the medium regardless of the growth phase of the bacterial culture. Cell associated enzyme was easily detached from the bacterial surface by treatment with a low concentration (0.05%) of Triton X-100. This procedure released more than 85-90% of activity in a soluble form. Subsequent acetone precipitation of proteins in the Triton X-100 fraction successfully separated the activity from pigment which remained in solution. The redissolved protein fraction, after dialysis, was applied to hydroxyapatite (100 ml) equilibrated with 20 mM potassium phosphate buffer pH 7.0. The elution was carried out with 20 mM potassium phosphate buffer pH 7.0, using a phosphate gradient from 20 mM to 300 mM at flow rate 20 ml/h. At this step substantial separation of the PTP-A activity from both the DPP IV and bulk protein was achieved (Fig. 1a). Further purification performed by subsequent chromatography steps including Phenyl-Sepharose (Fig. 1b), MonoQ (Fig. 1c) and MonoP columns (Fig. 1d), resulted in the isolation of purified enzyme.

Phenyl-Sepharose HP (25 ml) was equilibrated with 50 mM potassium phosphate, 1M ammonium sulfate, pH 7.0, at flow rate 30 ml/h. The column was

washed with two volumes of equilibration buffer and a step gradient of 0.5 M ammonium sulfate was applied, following which a descending gradient of 0.5 to 0 M ammonium sulfate was applied. The PTP-A containing fractions were extensively dialyzed against 20 mM Tris-HCl, pH 7.0, and concentrated by ultrafiltration. The concentrated PTP-A containing fractions were applied to a MonoQ column equilibrated with the same buffer. The column was washed with 5 volumes of equilibration buffer, following which bound protein was eluted with a gradient of 0-300 mM NaCl. The concentrated fraction of PTP-A from the MonoQ column was equilibrated with 25 mM Bis-Tris, pH 6.3, and loaded on a MonoP column equilibrated with the same buffer. A pH gradient was developed using 50 ml of Polybuffer 74, with the pH adjusted to 4.0.

Significantly, the chromatography step on the MonoP column yielded the A_{280} profile much sharper than the activity peak. Although this imperfect overlap of protein and activity may suggest that the protein component does not represent the active enzyme, the rest of data argues with such a contention. This apparent contradiction may be likely explained by the enzyme inhibition by the reaction product of H-Ala-Phe-Pro-pNA hydrolysis but this possibility has not been explored. The yield of protein and activity recovery in a typical purification procedure is summarized in Table 1.

Table 1. Purification of the PTP- A from *P. gingivalis*

Step	Volume (ml)	Protein (mg)	Total activity*	Specific activity (units/mg)	Purification fold	Yield (%)
Triton X-100 extract after centrifugation	200	1200	757 673	642	1	100
Acetone precipitate	50	600	537 622	896	1.4	71
Hydroxyapatite chromatography	50	22	400 039	18 183	28	53
Phenyl-Sepharose	48	10	312 505	31 250	48	41
MonoQ	3	1.5	244 828	163 218	254	32
MonoP	4	0.7	188 400	269 142	420	25

* Based on the enzymatic activity using H-Ala-Phe-Pro-pNA where one unit = mOD/min/1ml

SDS-PAGE analysis of the purified enzyme revealed the presence of two protein bands with apparent molecular masses of 81.8 and 75.8 kDa, respectively (Fig. 2, lane f). Autoradiography of the enzyme sample radiolabeled with [1,3-^{3}H]DFP (Fig. 2, lane g) clearly indicated that the bands represented either two distinct serine peptidases or different molecular mass forms of the same enzyme. In an attempt to distinguish between these two options, the electrophoretically resolved proteins were subjected to amino terminal sequence analysis. Unfortunately, it was found that the 81.8 kDa form of PTP-A had a blocked N-terminus. In contrast, the sequence NH_2-SAQTTRFSAADLNALMP (SEQ ID NO:23) was found at the N-terminus of the lower molecular mass form of the enzyme. This result led us to the possibility that the 75.8 kDa form of PTP-A was derived from the 81.8 kDa form through proteolytic cleavage of a 6 kDa amino-terminal peptide. To confirm this hypothesis and, in addition, to localize the active site residue within *P. gingivalis* PTP-A, the mixture containing both radiolabeled enzymes was proteolytically fragmented and peptides resolved by reverse-phase HPLC. This procedure yielded only one major radioactive peptide peak, and the purified peptide was found to have a single amino acid sequence: IGVHGWXYGGFMTTNL (SEQ ID NO:24), where X apparently represents the active-site serine residue covalently and irreversibly modified by DFP. These data convincingly indicate that the two protein bands of purified PTP-A represents different forms of the same enzyme. The portion of the purified PTP-A having a truncated N-terminus may be due to cleavage by Lys-specific peptidase and is likely to be an artifact which occurred during the purification procedure. Nevertheless, the proteolytic shedding of membrane bound PTP-A also occurs during cultivation of the bacteria, as indicated by variable amount of soluble activities found in cell free culture media.

Example 3

pH Optimum, Stability and Inhibition Profile

Using the amidolytic activity assay with H-Ala-Phe-Pro-pNA it was found that the enzyme has a broad pH optimum from pH 6.0 to 8.0 and in 0.2 M HEPES, pH 7.6 was stable for at least 12 hours at 25°C or 37°C. PTP-A activity was not affected by class specific synthetic inhibitors of cysteine or metalloproteinases

(Table 2). In contrast, preincubation of the enzyme with DFP or PEFABLOCK resulted in total loss of activity, supporting its classification as a serine peptidase. Surprisingly, however, 3,4-dichloroisocumarin was only a poor inhibitor, and PMSF, leupeptin, antipain and prolinal had no effect at all. Interestingly, preincubation of PTP-A with iodoacetamide, but not with N-ethylmaleimide, stimulated enzyme amidolytic activity about two-fold. Human plasma inhibitors, such as α_1-proteinase inhibitor, α_1-antichymotrypsin and α_2-macroglobulin did not affect the enzyme activity, nor were they cleaved by PTP-A.

The effect of inhibitors on amidolytic activity of DPP IV was also determined using the same conditions as those used for PTP-A, but using H-Gly-Pro-pNA as a substrate.

Table 2. Effect of inhibitors on the amidolytic activity of PTP-A and DPP IV. Results are for a 15-min incubation at 37 C in 0.2 HEPES pH 7.6, with 1 mM H-Ala-Phe-Pro-pNA as substrate.

Inhibitor	Concentration	Residual activity of PTP-A, %	Residual activity of DPP IV, %
Diisopropyl fluorophospate	10 mM	0	0
	10 mM	96	20
Phenylmethanesulfonyl fluoride	1mg/ml	20	15
	10mg/ml	0	0
PEFABLOC SC	1 mM	56	100
3,4-dichloroisocoumarin	5mM	200	100
Iodoacetamide	5 mM	100	100
N-Ethylmaleimide	1 mM	98	100
1,10-orthophenanthroline	5 mM	93	100
	0.1 mM	100	100
EDTA	0.1 mM	100	100
Leupeptin	0.1 mM	100	20
Antipain	10 mM	100	0
Prolinal	10 mM	100	30
Val-Pro	10 mM	100	1
Ala-Pro			
Ala-Gly-Pro			

Example 4

Substrate Specificity

Among several chromogenic substrates tested, including H-Ala-Phe-Pro-pNA, H-Gly-Pro-pNA, Z-Gly-Pro-pNA, Z-Ala-Pro-pNA, H-Pro-pNA, only H-Ala-Phe-Pro-pNA was hydrolyzed by PTP-A indicating a prolyl specific tripeptidyl-peptidase activity. To further confirm this specificity several synthetic peptides composed of 5 to 34 amino acid residues and containing at least one proline residue were tested as substrates for PTP-A. Out of 22 peptides tested only those with a proline residue in the third position from the amino terminal end were cleaved (Table 3), with the significant exception of peptides with adjacent proline residues (peptides 3, 4 and 16). In addition, a free α-amino group was absolutely required for cleavage after the third proline residue as exemplified by resistance to enzymatic hydrolysis of peptide 9, which differs from the peptide 8 only in acylation of the α-amino group of the N-terminal valine residue. Except for these two limitations, the peptide bond -Pro-↓-Yaa- was cleaved at the same rate in all peptides with the general formula NH_2-Xaa-Xaa-Pro-Yaa-$(Xaa)_n$ (SEQ ID NO:25), where Xaa represents any amino acid residue while Yaa could be any residue except proline, regardless of the chemical nature of the amino acids and the length of the peptide. In all cases the reaction was completed within 3 hours and prolonged incubation for 24 hours did not affect the pattern of cleavage, confirming the absolute requirement for a proline residue at the third position from the unblocked N-terminus. In addition, these data indicate that the preparation of PTP-A was free of any contamination with either aminopeptidase, dipeptidyl peptidase, or endopeptidase activities.

The cleavage specificity of DPP IV was also determined using the same conditions as those used for PTP-A. The results (Table 3) demonstrate that DPP IV does not cleave between two proline residues.

Table 3. Cleavage specificity of PTP-A and DPP IVon synthetic peptides.

Substrate	Cleavage site	SEQ ID NO:
Peptide 1	H-Arg-Pro-**Pro**-↓-Gly-Phe-Ser-**Pro**-Phe-Arg	1
Peptide 2	H-Arg-**Pro**-**Pro**-↓-Gly-Phe	2
Peptide 3	H-Lys-Arg-**Pro**-**Pro**-Gly-Phe-Ser-**Pro**-Phe-Arg	3
Peptide 4	H-Tyr-Arg-**Pro**-**Pro**-Gly-Phe-Ser-**Pro**-Phe-Arg	4
Peptide 5	H-Arg-**Pro**-**Hyp**-Gly-Phe-Ser-**Pro**-Phe-Arg	5
Peptide 6	H-Arg-**Pro**-↑-Lys-**Pro**-↑-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2	6
Peptide 7	H-Val-**Pro**-**Pro**-↓-Gly-Glu-Asp-Ser-Lys-Glu-Val-Ala-Ala-**Pro**-His-Arg-Gln	7
Peptide 8	H-Val-**Pro**-**Pro**-↓-Gly-Glu-Asp-Ser-Lys	8
Peptide 9	Ac-Val-**Pro**-**Pro**-Gly-Glu-Asp-Ser-Lys	9
Peptide 10	H-Val-Glu-**Pro**-↓-Ile-**Pro**-Tyr	10
Peptide 11	H-Arg-Gly-**Pro**-↓-Phe-**Pro**-Ile	11
Peptide 12	H-Ala-Arg-**Pro**-↓-Ala-D-Lys-amide	
Peptide 13	H-**Pro**-Asn-**Pro**-↓-**Asn**-Gln-Gly-Asn-Phe-Ile	13
Peptide 14	H-Arg-His-**Pro**-↓-Lys-Tyr-Lys-Thr-Glu-Leu	14
Peptide 15	H-Gly-Val-**Pro**-↓-Lys-Thr-His-Leu-Glu-Leu	15
Peptide 16	H-Lys-Gly-**Pro**-**Pro**-Ala-Ala-Leu-Thr-Leu	16
Peptide 17	H-Gln-Lys-Gln-Met-Ser-Asp-Arg-Arg-Glu-Asn-Asp-Met-Ser-**Pro**-Ser-Asn-Asn-Val-Val-**Pro**-Ile-His-Val-**Pro**-**Pro**-Thr-Thr-Glu-Asn-Lys-**Pro**-Lys-Val-Gln	17
Peptide 18	H-Phe-Leu-Arg-Glu-**Pro**-Val-Ile-Phe-Leu	18
Peptide 19	H-Gly-Ile-Arg-**Pro**-Tyr-Glu-Ile-Leu-Ala	19
Peptide 20	H-Leu-**Pro**-↑-Asp-Leu-Asp-Ser-Ser-Leu-Ala-Ser-Ile-Gln-Glu-Leu-Leu-Ser-**Pro**-Gln-Glu-**Pro**--**Pro**-Arg-**Pro**-**Pro**-Glu-Ala	20
Peptide 21	H-Cys-Leu-Ser-Ser-Gly-Thr-Leu-**Pro**-Gly-**Pro**-Gly-Asn-Asp-Ala-Ser-Arg-Glu-Leu-Glu-Ser	21
Peptide 22	H-Lys-Ile-**Ala**-Gly-Tyr-His-Leu-Glu-Leu	22
Peptide 23	H-Ser-**Pro**-↑-Tyr-Ser-Ser-Asp-Thr-Thr	46
Peptide 24	H-Ala-**Pro**-↑-Val-Arg-Ser-Leu-Asn-Cys-Thr-Leu-Arg-Asp-Ser-Gln-Gln-Lys	47

↓ indicates cleavage site mediated by PTP-A

↑ indicates cleavage site mediated by DPP IV

The lack of cleavage after internal proline residues in the synthetic peptides corresponds well with the absence of any proteolytic activity on several protein substrates including IgA, IgG, albumin, azocasein, carboxymethylated rybonuclease and gelatin. However, the size of substrate, which is a limiting factor in the activity of oligopeptidases (Walter, R., et al., (1980) *Mol. Cell. Biochem.* 30, 111-126), is not restricting in the case of PTP-A, because the enzyme is able to cleave a tripeptide (NH_2-Xaa-Xaa-Pro) from the N-terminus of both human cystatin C and interleukin 6.

Example 5

PTP-A Sequence Analysis

Partial PTP-A amino acid sequence data allowed us to identify the *P. gingivalis* genomic clone gnl | TIGR | P. gingivalis_126 in the Unfinished Microbial Genomes data base, TIGR. An ORF corresponding to the PTP-A amino acid sequence was found as indicated by the fact that all sequences of the PTP-A derived peptides obtained by the enzyme polypeptide fragmentation with RgpB were present in the protein primary structure inferred from the nucleotide sequence of the ORF. The 732 amino acid polypeptide with a calculated mass of 82, 266 Da was encoded in this ORF. The homology search performed using the NCBI TBLASTN tool against GenBank+EMBL+DDBJ+PDB databases and subsequent multiple sequence alignments using the ClustalW Multiple Sequence Alignment tool (Fig. 3) indicated that PTP-A is a new member of the prolyl oligopeptidase (S9) family of serine peptidases (Rawlings, N.D., et al., (1991) *Biochem. J.* 279, 907-908).

The sequence GXSXGG (SEQ ID NO:40) is a signature feature for the S9 family of serine peptidases. Within this large and diverse S9 family of evolutionary and functionally related enzymes both from prokaryotes and eukaryotes, PTP-A was most closely related to bacterial dipeptidyl peptidase IV (DPP IV) from *Flavobacterium meningosepticum*, *Xantomonas maltophilus*, and *P. gingivalis*, sharing 31.6%, 30.4%, and 28.5% amino acid sequence identity, respectively. Remarkably, the COOH-terminal region of the PTP-A molecule (residues 502 - 732) shows a significant similarity to the eukaryotic prolyl oligopeptidases with 34% and 33% identity to human DPP IV and mouse fibroblast activation protein (FAP), respectively (Fig. 3). This part of the molecule contains the amino acid

residues which encompass the catalytic triad in all characterized prolyl oligopeptidases, and from the multiple alignments with DPP IV of confirmed active site residues (Kabashima,T., et al., (1995) *Arch. Biochem. Biophys.* 320, 123-128) it is apparent that Ser-603, Asp-678 and His-710 represent the catalytic triad of PTP-A (Fig. 3). Such an inference is further supported by the direct labeling of Ser-603 by DFP. In addition, the computer assisted search for sequential motifs characteristic for transmembrane domains revealed the presence of such a putative region within the N-terminal sequences of PTP-A, with residues 5 to 25 most likely folded into a hydrophobic α-helix responsible for membrane anchoring of this enzyme.

In *P. gingivalis* PTP-A, as well as in DPP IV, all activities are cell surface associated, and it is conceivable that the enzymes are membrane anchored through putative signal sequences which are not cleaved but remain as a membrane spanning domain similar to other members of the prolyl oligopeptidase family. The cell surface localization of di- and tripeptidyl-peptidases suggests a putative physiological function in providing nutrients for growing bacterial cells. Here, the inability of asaccharolytic *P. gingivalis* to utilize free amino acids (Dashper, S.G., et al., *J. Dent Res.* 77 ,1133 (Abstract) (1988)) makes the bacterium entirely dependant on an external peptide supply. In this regard, DPP-IV and PTP-A activities are probably very important, if not indispensable, for bacterial growth.

This suggestion is strongly corroborated by the fact that the *P. gingivalis* genome contains three additional genes encoding peptidases homologous with DPP-IV and PTP-A and one related to aminopeptidase B. The peptidases homologous with DPP-IV and PTP-A are referred to as homologs H1 (SEQ ID NO:43), H2 (SEQ ID NO:44), and H3 (SEQ ID NO:45) (Fig. 6). If expressed, each gene product would probably have enzymatic activity because each has a well preserved catalytic triad (Fig. 4). In addition, all of these genes encode a putative signal peptide which may act in providing membrane-anchorage motifs.

Example 6

Influence of Proteinase Inhibitor on *P. gingivalis* Growth

To evaluate whether *P. gingivalis* growth was influenced by the presence of a peptidase inhibitor, *P. gingivalis* in logphase growth was diluted 1:5 into liquid media and incubated at 37°C. The cell density was monitored by measuring the optical density at 600 nm (OD_{600}). When the optical density began to increase, Pefabloc was added at 0.5 mg/ml or at 2.0 mg/ml. The control culture received no Pefabloc. The cultures receiving Pefabloc exhibited decreased growth (Figure 5). The peptidase inhibitor had to be added before the culture reached an OD_{600} of about 0.3 for the peptidase inhibitor to have an effect on growth.

The complete disclosures of all patents, patent applications, publications, and nucleic acid and protein database entries, including for example GenBank accession numbers and EMBL accession numbers, that are cited herein are hereby incorporated by reference as if individually incorporated. Various modifications and alterations of this invention will become apparent to those skilled in the art without departing from the scope and spirit of this invention, and it should be understood that this invention is not to be unduly limited to the illustrative embodiments set forth herein.

Sequence Listing Free Text

SEQ ID NOs:1-11:	Synthetic peptides
SEQ ID NO:12:	Target peptide
SEQ ID NOs:13-22:	Synthetic peptides
SEQ ID NO:23:	Amino-terminus of the lower molecular mass form of PTP-A.
SEQ ID NO:24:	Amino acid sequence present in PTP-A, where X apparently represents the active-site serine residue covalently and irreversibly modified by DFP.
SEQ ID NO:25:	Target peptide, where Xaa represents a natural or modified amino acid residue, Yaa represents a natural or modified amino acid residue except proline, and N is equal to or greater than 1.
SEQ ID NO:26:	Mouse fibroblast activation protein

SEQ ID NO:27:	Human DPP IV
SEQ ID NO:28:	DPP from *Flavobacterium meningosepticum*
SEQ ID NO:29:	DPP from *P. gingivalis*
SEQ ID NO:30:	*P. gingivalis* PTP-A
SEQ ID NO:31:	Portion of PTP-A
SEQ ID NO:32:	Portion of DPP from *P. gingivalis*
SEQ ID NO:33:	Portion of H1 homolog of *P. gingivalis* DPP
SEQ ID NO:34:	Portion of H2 homolog of *P. gingivalis* DPP
SEQ ID NO:35:	Portion of H3 homolog of *P. gingivalis* DPP
SEQ ID NOs:36-37:	Probes
SEQ ID NO:38:	Nucleotide sequence of coding region encoding PTP-A.
SEQ ID NO:39:	Consensus sequence for clan SC where X is any amino acid and S is the active site serine GXSXXG.
SEQ ID NO:40:	Consensus sequence for family S9 where X is any amino acid and S is the active site serine GXSXGG.
SEQ ID NO:41:	A specific substrate for a prolyl-tripeptidyl peptidase, where Xaa represents a natural or modified amino acid residue, and Yaa represents a natural or modified amino acid residue except proline.
SEQ ID NO:42:	DPP from *P. gingivalis*
SEQ ID NO:43:	H1 homolog of *P. gingivalis* DPP
SEQ ID NO:44:	H2 homolog of *P. gingivalis* DPP
SEQ ID NO:45:	H3 homolog of *P. gingivalis* DPP
SEQ ID NO:46:	Synthetic peptides
SEQ ID NO:47:	Synthetic peptides
SEQ ID NO:48:	Amino terminal sequence of DPP IV

What is claimed is:

1. An isolated prolyl tripeptidyl-peptidase, active analog, active fragment, or active modification thereof having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids, wherein the prolyl tripeptidyl-peptidase:target polypeptide ratio is at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours.

2. An isolated prolyl tripeptidyl-peptidase, active analog, active fragment, or active modification thereof having amidolytic activity at a prolyl tripeptidyl-peptidase:target polypeptide ratio of at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours, and wherein the prolyl tripeptidyl-peptidase is isolated from *P. gingivalis*.

3. The isolated prolyl tripeptidyl-peptidase of claim 2 wherein the peptide cleaved by the isolated prolyl tripeptidyl-peptidase comprises the sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:11, H-Ala-Arg-Pro-Ala-D-Lys-amide, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:25, and SEQ ID NO:37.

4. The isolated prolyl tripeptidyl-peptidase of claim 2 wherein the amino acid sequence of the isolated prolyl tripeptidyl-peptidase comprises the amino acid sequence GXSXXG (SEQ ID NO:39).

5. The isolated prolyl tripeptidyl-peptidase of claim 4 wherein the amino acid sequence of the isolated prolyl tripeptidyl-peptidase comprises the amino acid sequence GXSXGG (SEQ ID NO:40).

6. The isolated prolyl tripeptidyl-peptidase of claim 4 wherein the amino acid sequence of the isolated prolyl tripeptidyl-peptidase comprises SEQ ID NO:30.

7. An isolated polypeptide, active analog, active fragment, or active modification thereof having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids, wherein the polypeptide:target polypeptide ratio of at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours.

8. An isolated polypeptide comprising an amino acid sequence having a percentage amino acid identity of greater than 35 % with SEQ ID NO:30.

9. An isolated nucleic acid fragment encoding a prolyl-tripeptidyl peptidase, active analog, active fragment, or active modification thereof, having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids, wherein the prolyl tripeptidyl-peptidase:target polypeptide ratio of at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours.

10. The nucleic acid fragment of claim 9 wherein the nucleic acid fragment has a nucleotide sequence comprising SEQ ID NO:38.

11. The nucleic acid fragment of claim 9 wherein a complement of the nucleic acid fragment hybridizes to SEQ ID NO:38 under hybridization conditions of 0.5 M phosphate buffer, pH 7.2, 7 % SDS, 10 mM EDTA, at 68°C, followed by three for 20 minutes washes in 2x SSC, and 0.1 % SDS, at 65°C, wherein at least about 20 nucleotides of the complement hybridize.

12. An isolated nucleic acid fragment encoding a polypeptide wherein the polypeptide comprises an amino acid sequence having a percentage amino acid identity of greater than 35 % with SEQ ID NO:30.

13. A method of identifying an inhibitor of a prolyl-tripeptidyl peptidase, active analog, active fragment, or active modification thereof, comprising identifying a molecule that inhibits the amidolytic activity of the prolyl-

tripeptidyl peptidase by incubating the prolyl-tripeptidyl peptidase with the molecule under conditions that promote amidolytic activity of the prolyl-tripeptidyl peptidase and determining if the amidolytic activity of the prolyl-tripeptidyl peptidase is inhibited relative to the amidolytic activity in the absence of molecule.

14. A method of reducing growth of a bacterium comprising inhibiting a prolyl tripeptidyl-peptidase, active analog, active fragment, or active modification thereof, by contacting the prolyl tripeptidyl-peptidase with an inhibitor of the prolyl tripeptidyl-peptidase.

15. A method for protecting an animal from a periodontal disease caused by *P. gingivalis* comprising administering to the animal the inhibitor of claim 14 wherein the disease is selected from the group consisting of gingivitis and periodontitis.

16. The method of claim 15 wherein the inhibitor is administered by a method selected from the group consisting of subgingival application and controlled release delivery.

17. A method of reducing growth of a bacterium comprising inhibiting a prolyl dipeptidyl-peptidase, active analog, active fragment, or active modification thereof, by contacting the prolyl dipeptidyl-peptidase with an inhibitor of the prolyl dipeptidyl-peptidase.

18. An immunogenic composition comprising an isolated prolyl tripeptidyl-peptidase, or an antigenic analog, antigenic fragment, or antigenic modification thereof, the prolyl tripeptidyl-peptidase having amidolytic activity for cleavage of a peptide bond present in a target peptide having at least 4 amino acids, wherein the prolyl tripeptidyl-peptidase:target polypeptide ratio is at least about 1:1 to no greater than about 1:10,000,000 in about 200 mM HEPES, about pH 7.5 at 37°C for at least about 3 hours.

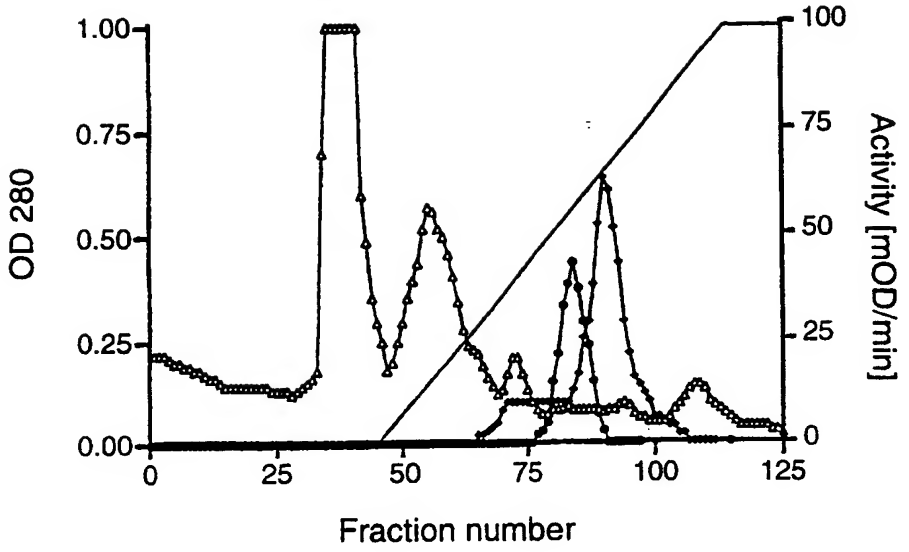
19. The immunogenic composition of claim 18 further comprising an adjuvant.

20. A composition comprising an inhibitor of an isolated prolyl tripeptidyl-peptidase and a pharmaceutically acceptable carrier.

21. A dipeptidyl peptidase having an amino acid sequence comprising SEQ ID NO:43.

22. A dipeptidyl peptidase having an amino acid sequence comprising SEQ ID NO:44.

23. A dipeptidyl peptidase having an amino acid sequence comprising SEQ ID NO:45.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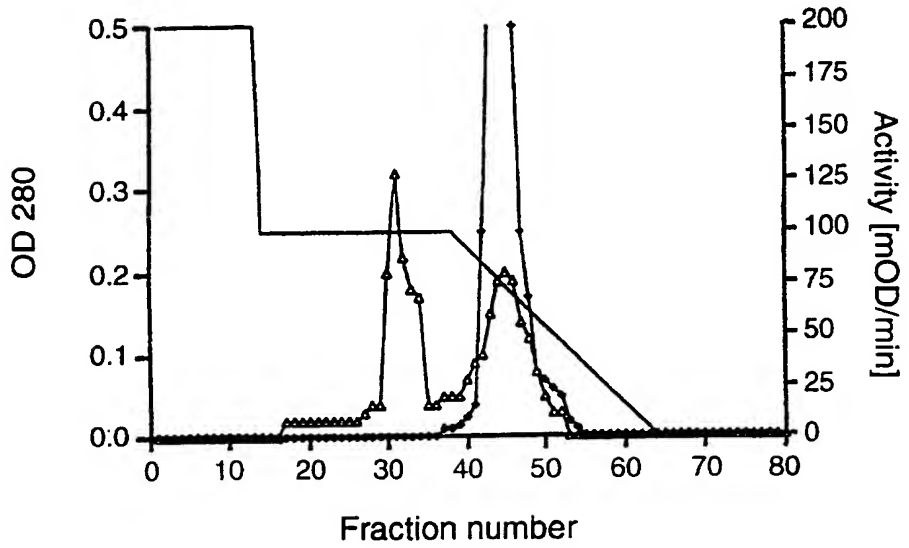
Activity [mOD/min]

Fig. 1a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0.2
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40
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Fraction number
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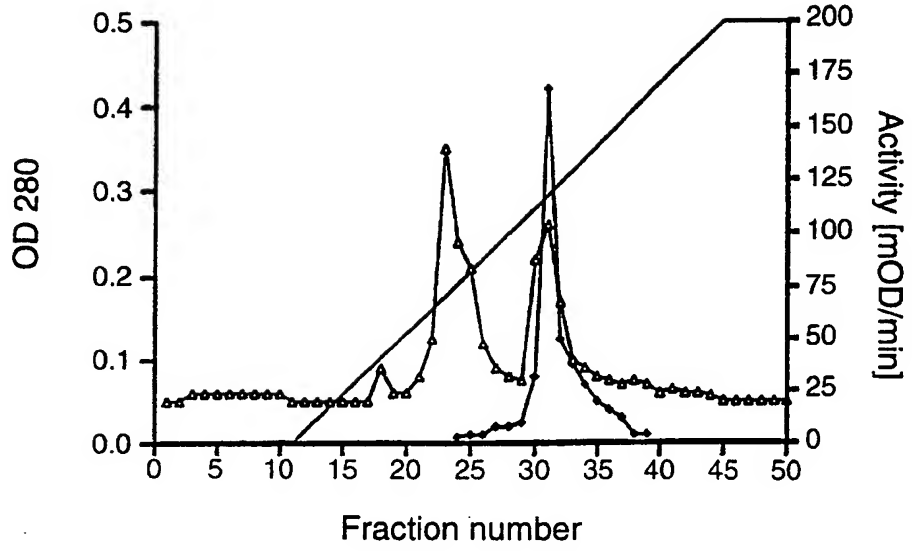
Activity [mOD/min]

Fig. 1b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Activity [mOD/min]
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30
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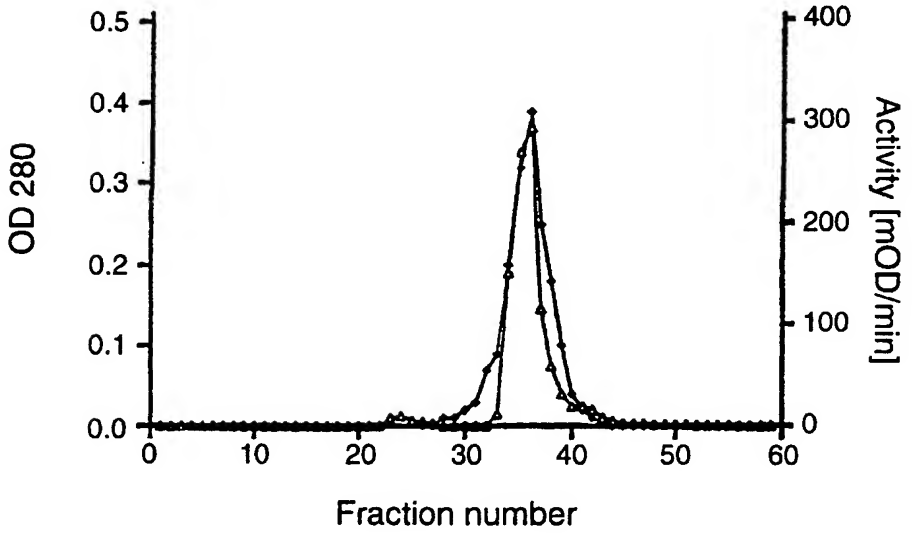
40
45
50

Fig. 1c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Activity [mOD/min]
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50
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Fig. 1d

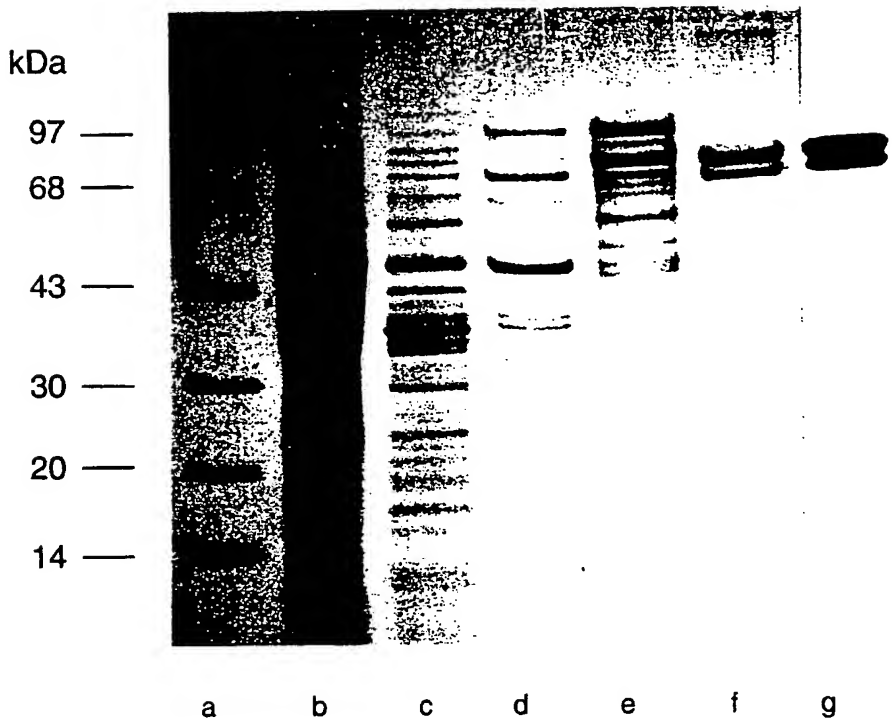


Fig. 2

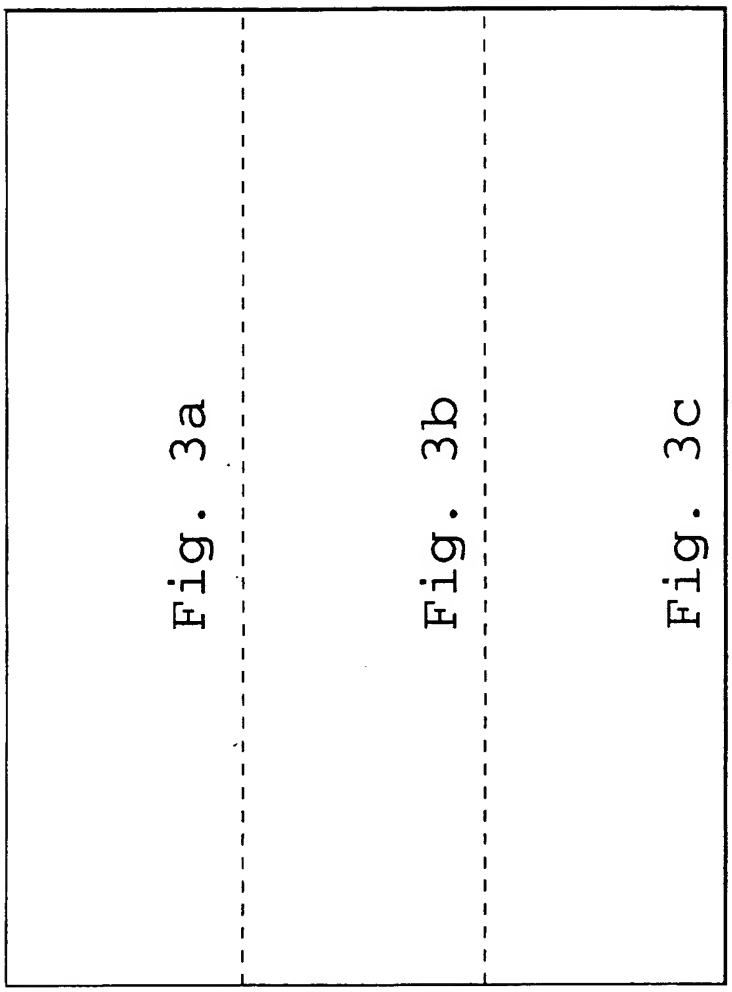


Fig. 3

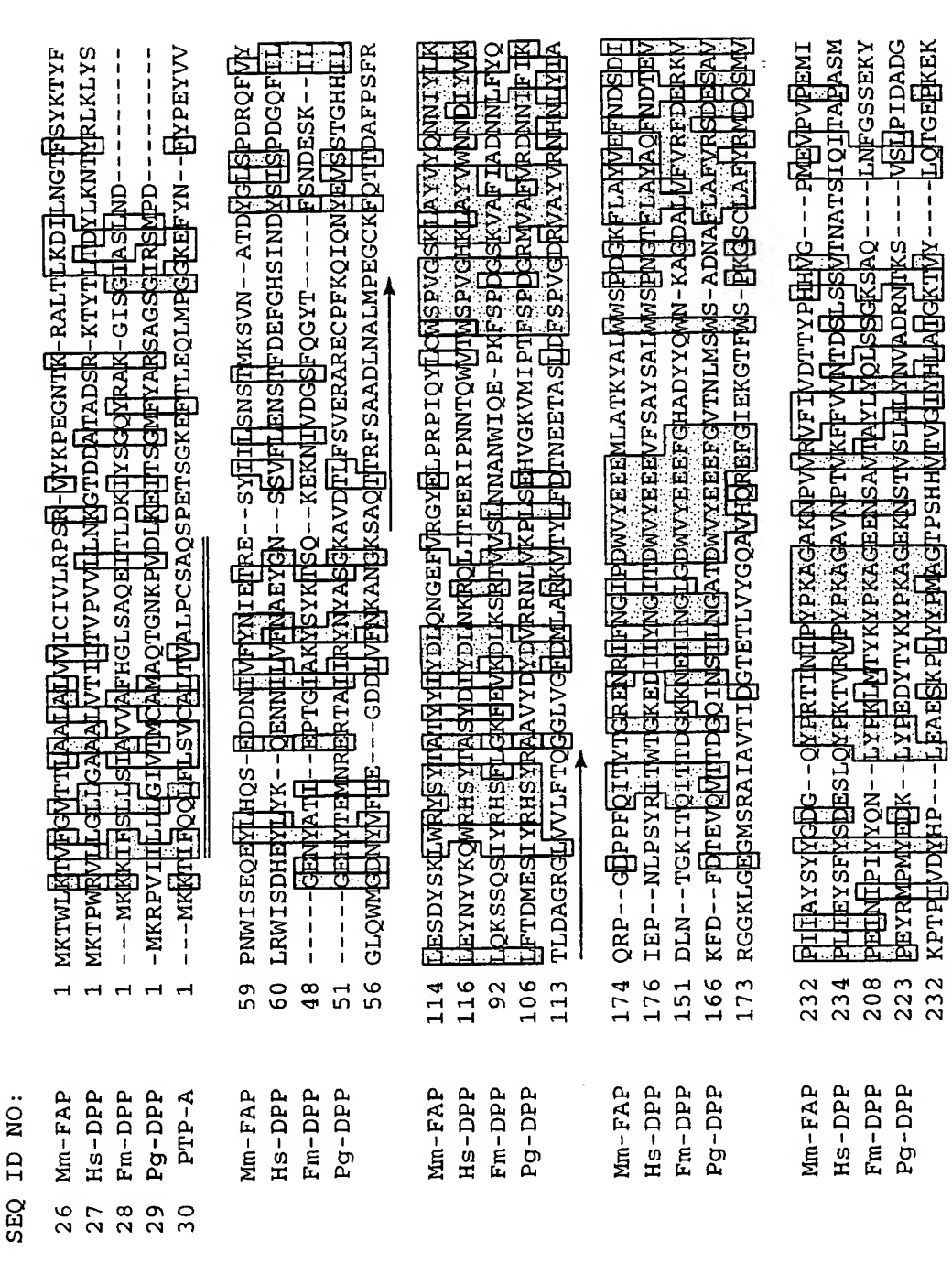
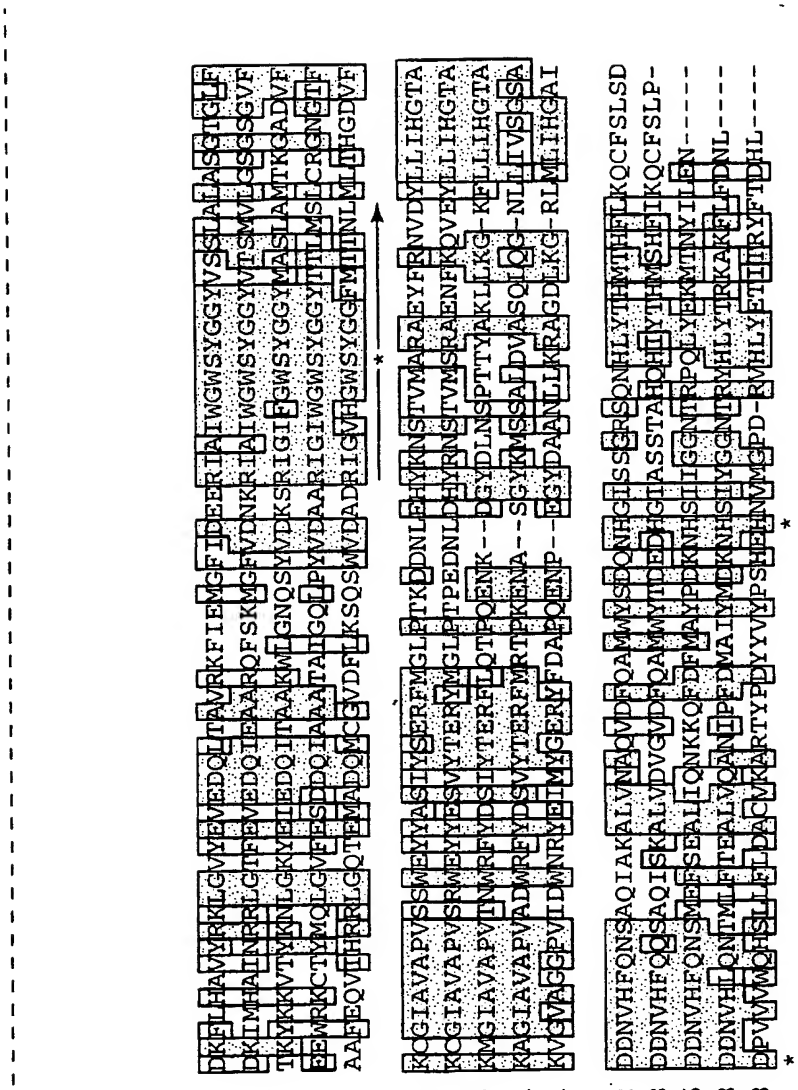


Fig. 3a

Fig. 3b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Fm-DPP
Pg-DPP
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611
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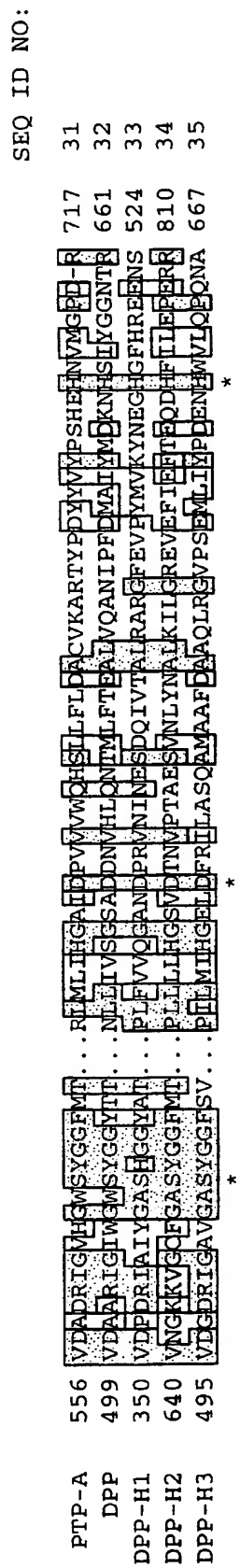
678

Fig. 3C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DPP 499 32
DPP-H1 350 33
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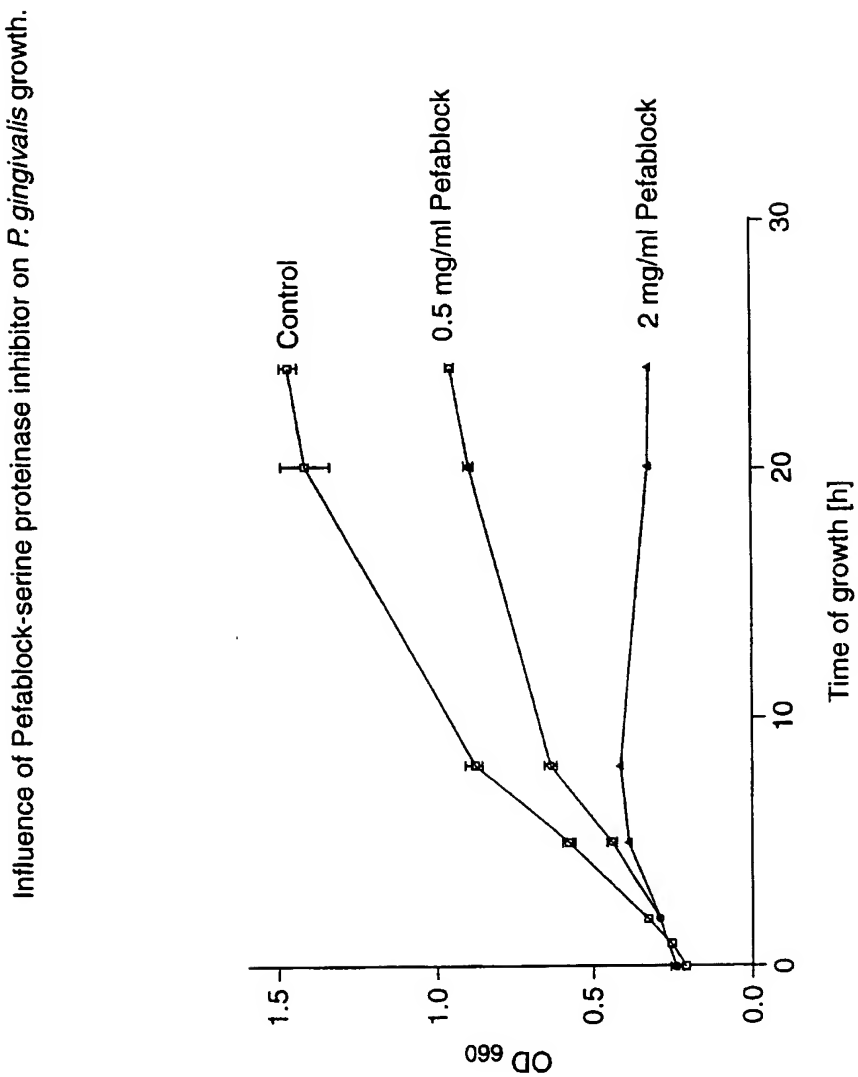
Fig. 4


Influence of Pefablock-serine proteinase inhibitor on P. gingivalis growth.
Control
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2 mg/ml Pefablock
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1.0
0.5
0.0
0
10
20
30
Time of growth [h]

Fig. 5

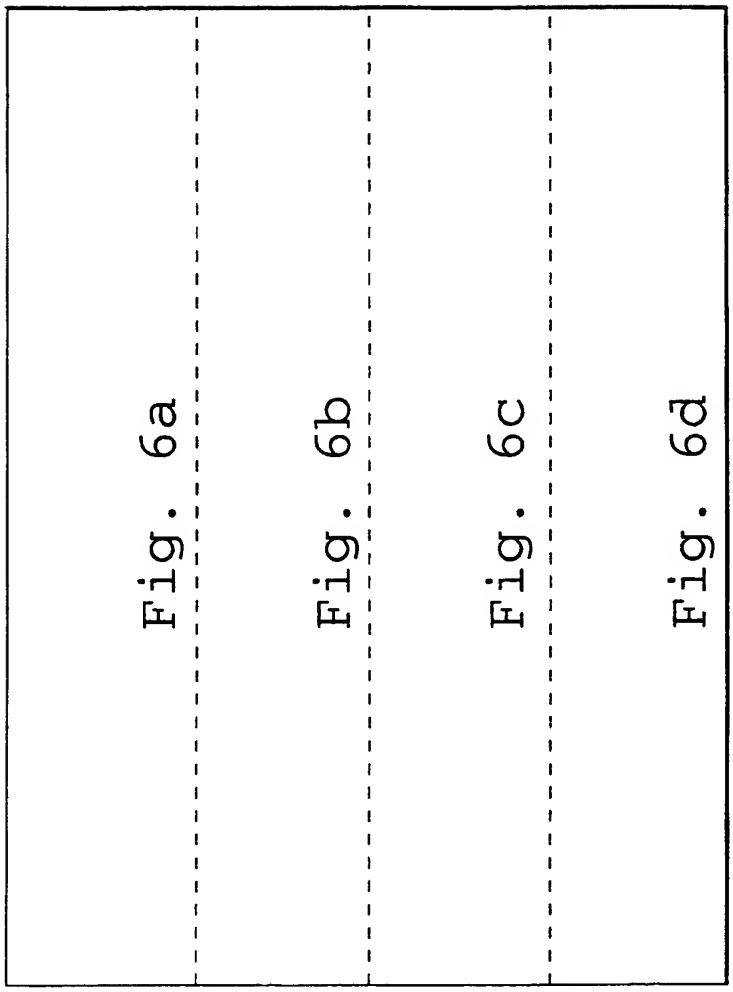

Fig. 6

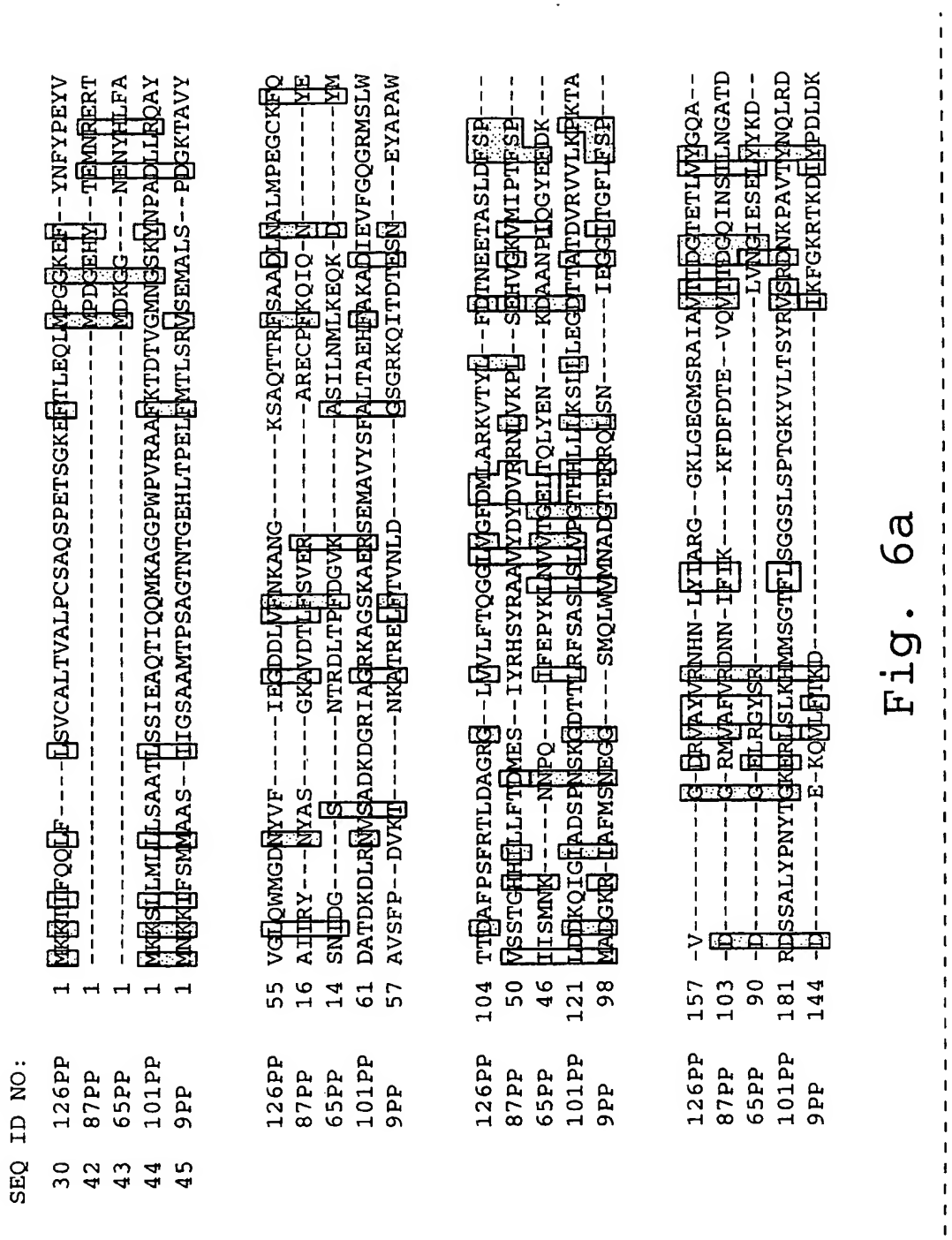

Fig. 6a

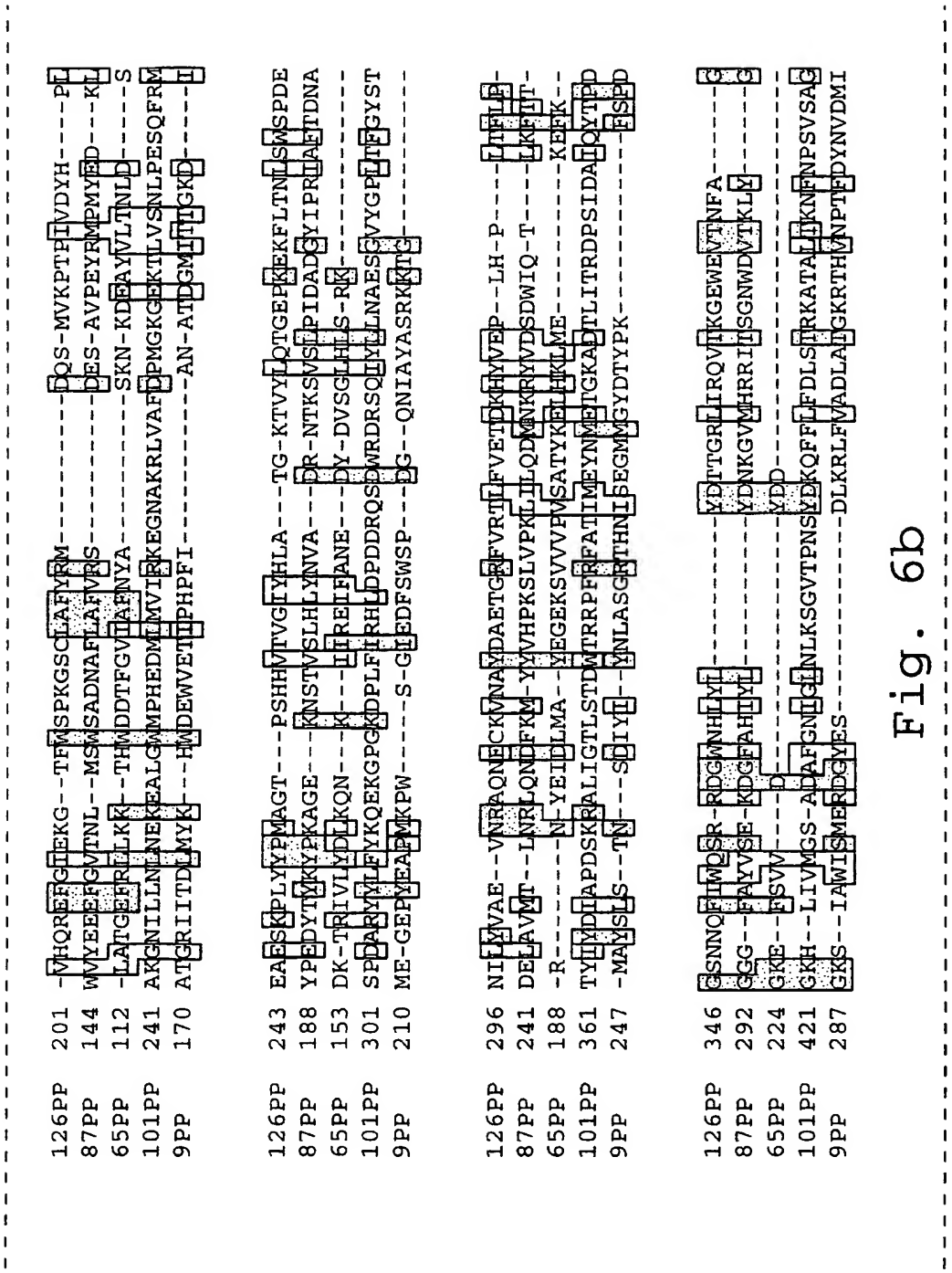

Fig. 6b

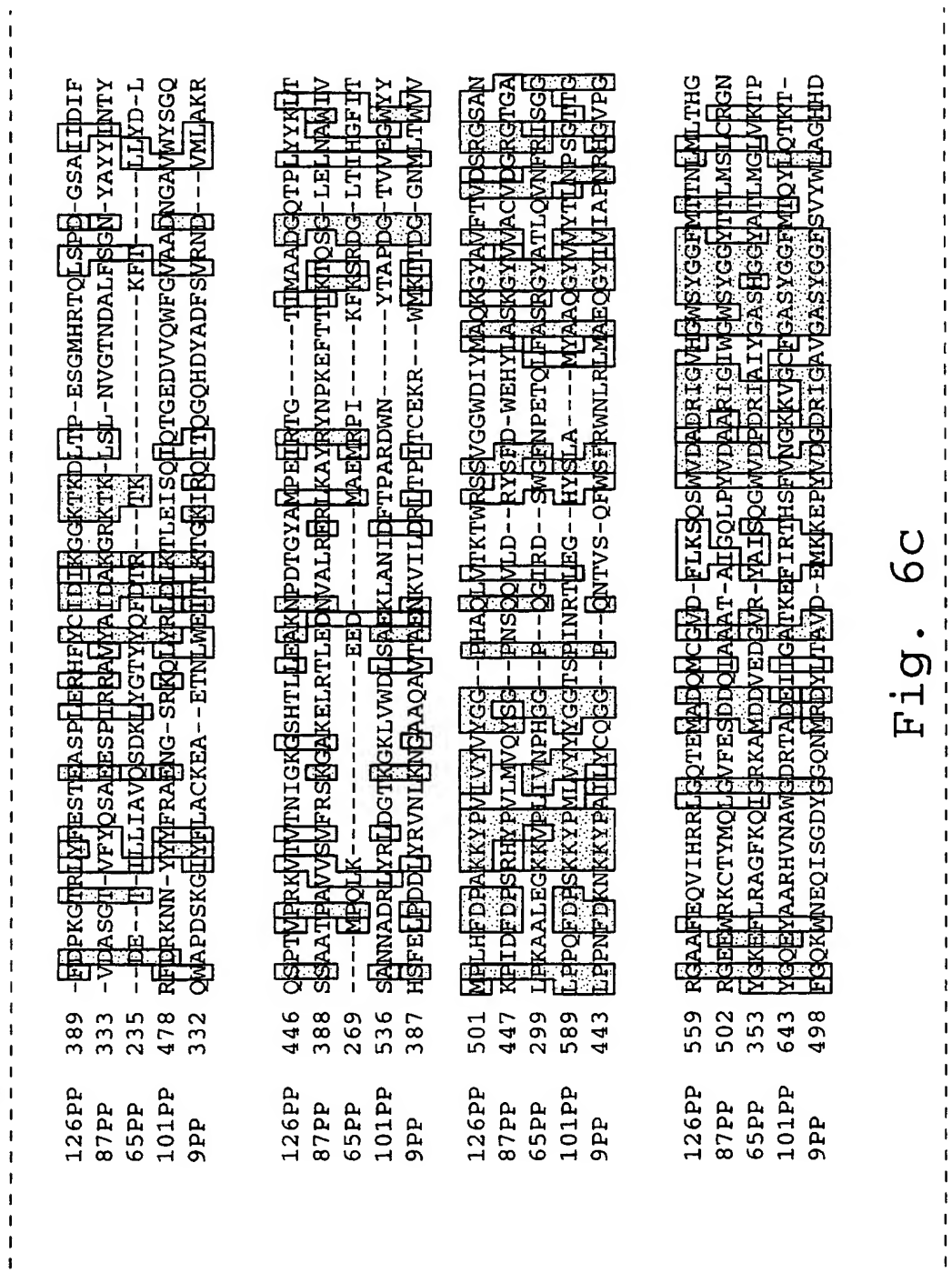

Fig. 6c

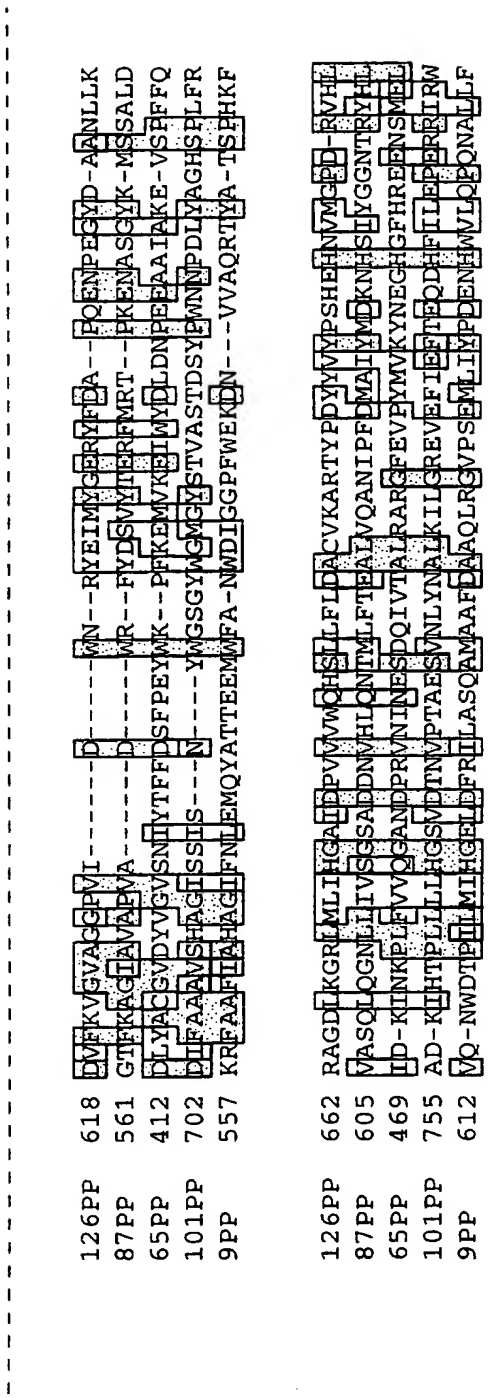

Fig. 6d

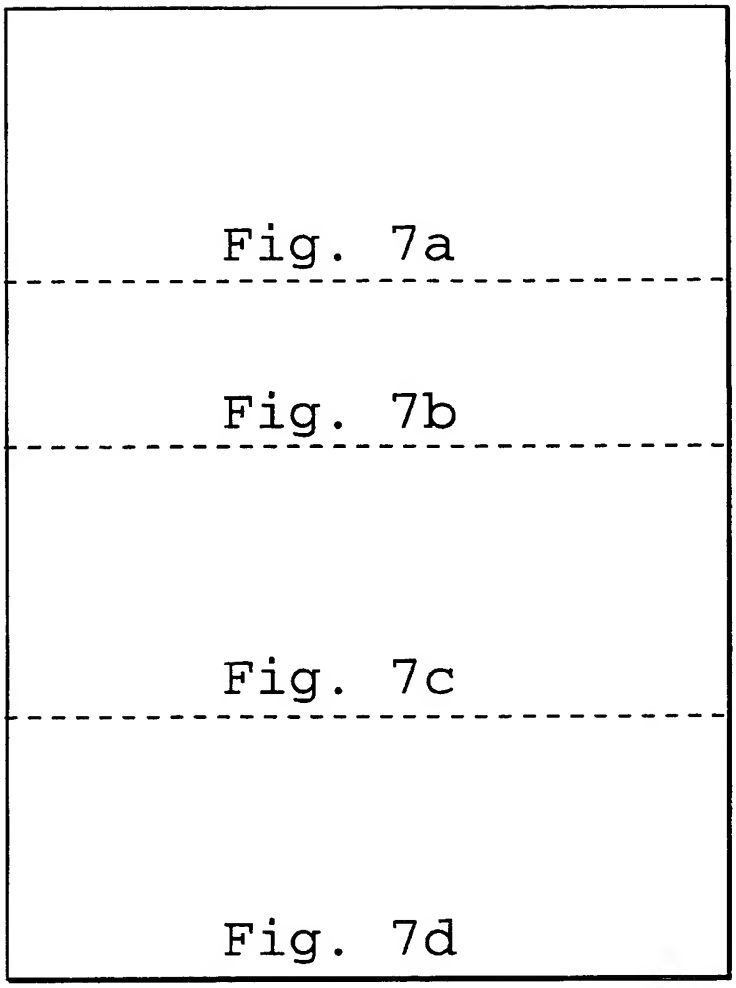

Fig. 7

P. gingivalis W 83 PTP sequence

SEQ ID NO: 38 13228 atgaagaagacaatcttccaacaactatttctgtctgtttgtgcc
SEQ ID NO: 30 M K K T I F Q Q L F L S V C A
13273 cttacagtggccttgccttgttcggctcagtctcctgaaacgagt
L T V A L P C S A Q S P E T S
13318 ggtaaggagtttactcttgagcaactgatgcccggaggaaaagag
G K E F T L E Q L M P G G K E
13363 ttttataacttttaccccgaatacgtggtcggtttgcaatggatg
F Y N F Y P E Y V V G L Q W M
13408 ggagacaattatgtctttatcgagggtgatgatttagtttttaat
G D N Y V F I E G D D L V F N
13453 aaggcgaatggcaaatcggctcagacgaccagattttctgctgcc
K A N G K S A Q T T R F S A A
13498 gatctcaatgcactcatgccggagggatgcaaatttcagacgact
D L N A L M P E G C K F Q T T
13543 gatgctttcccttcattccgcacactcgatgccggacggggactg
D A F P S F R T L D A G R G L
13588 gtcgttctatttacccaaggaggattagtcggattcgatatgctt
V V L F T Q G G L V G F D M L
13633 gctcgaaaggtgacttatcttttcgataccaatgaggagacggct
A R K V T Y L F D T N E E T A
13678 tctttggatttttctcctgtgggagaccgtgttgcctatgtcaga
S L D F S P V G D R V A Y V R
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N H N L Y I A R G G K L G E G
13768 atgtcacgagctatcgctgtgactatcgatggaactgagactctc
M S R A I A V T I D G T E T L
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V Y G Q A V H Q R E F G I E K
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13903 atggatcagagtatggtgaagcctaccccgatagtggattatcat
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P L E A E S K P L Y Y P M A G
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D A E T G R F V R T L F V E T
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D K H Y V E P L H P L T F L P

Fig. 7a

```
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      S  A  N  R  G  A  A  F  E  Q  V  I  H  R  R
14938 ttggggcagaccgagatggccgatcagatgtgcggtgtggatttc
      L  G  Q  T  E  M  A  D  Q  M  C  G  V  D  F
14983 ctcaagagccaatcatgggtggatgccgatagaataggagtacat
      L  K  S  Q  S  W  V  D  A  D  R  I  G  V  H
15028 ggctggagctatggtggctttatgactacgaatctgatgcttacg
      G  W  S  Y  G  G  F  M  T  T  N  L  M  L  T
15073 cacggcgatgtcttcaaagtcggagtagccggcgggcctgtcata
      H  G  D  V  F  K  V  G  V  A  G  G  P  V  I
15118 gactggaatcgatatgagattatgtacggtgagcgttatttcgat
      D  W  N  R  Y  E  I  M  Y  G  E  R  Y  F  D
15163 gcgccacaggaaaatcccgaaggatacgatgctgccaacctgctc
      A  P  Q  E  N  P  E  G  Y  D  A  A  N  L  L
15208 aaacgagccggtgatctgaaaggacgacttatgctgattcatgga
      K  R  A  G  D  L  K  G  R  L  M  L  I  H  G
15253 gcgatcgatccggtcgtggtatggcagcattcactccttttccttt
```

Fig. 7b

```
      A  I  D  P  V  V  V  W  Q  H  S  L  L  F  L
15298 gatgcttgcgtgaaggcacgcacctatcctgactattacgtctat
      D  A  C  V  K  A  R  T  Y  P  D  Y  Y  V  Y
15343 ccgagccacgaacataatgtgatggggccggacagagtacatttg
      P  S  H  E  H  N  V  M  G  P  D  R  V  H  L
15388 tatgaaacaataacccgttatttcacagatcacttatga 15426
      Y  E  T  I  T  R  Y  F  T  D  H  L  *
```

Fig. 7c

SEQ ID NO:38

ATGAAGAAGACAATCTTCCAACAACTATTTCTGTCTGTTTGTGCCCTTACAGTGGCCTTGCCTTGTTCGGC
TCAGTCTCCTGAAACGAGTGGTAAGGAGTTTACTCTTGAGCAACTGATGCCCGGAGGAAAAGAGTTTTATA
ACTTTTACCCCGAATACGTGGTCGGTTTGCAATGGATGGGAGACAATTATGTCTTTATCGAGGGTGATGAT
TTAGTTTTTAATAAGGCGAATGGCAAATCGGCTCAGACGACCAGATTTTCTGCTGCCGATCTCAATGCACT
CATGCCGGAGGGATGCAAATTTCAGACGACTGATGCTTTCCCTTCATTCCGCACACTCGATGCCGGACGGG
GACTGGTCGTTCTATTTACCCAAGGAGGATTAGTCGGATTCGATATGCTTGCTCGAAAGGTGACTTATCTT
TTCGATACCAATGAGGAGACGGCTTCTTTGGATTTTTCTCCTGTGGGAGACCGTGTTGCCTATGTCAGAAA
CCATAACCTTTACATTGCTCGTGGAGGTAAATTGGGAGAAGGTATGTCACGAGCTATCGCTGTGACTATCG
ATGGAACTGAGACTCTCGTATATGGCCAGGCCGTACACCAGCGTGAATTCGGTATCGAAAAAGGTACATTC
TGGTCTCCAAAAGGGAGCTGCCTTGCTTTCTATCGAATGGATCAGAGTATGGTGAAGCCTACCCCGATAGT
GGATTATCATCCGCTCGAAGCCGAGTCCAAACCGCTTTATTACCCCATGGCAGGTACTCCGTCACACCACG
TTACGGTTGGGATCTATCATCTGGCCACAGGTAAGACCGTCTATCTACAAACGGGTGAACCCAAGGAAAAA
TTTCTGACGAATTTGAGTTGGAGTCCGGACGAAAATATCTTGTATGTAGCTGAGGTGAATCGTGCTCAAAA
CGAATGTAAGGTAAATGCCTATGACGCTGAGACCGGTAGATTCGTCCGTACGCTTTTTGTTGAAACCGATA
AACATTATGTAGAGCCGTTACATCCCCTGACATTCCTTCCGGGAAGTAACAATCAGTTCATTTGGCAGAGC
CGTCGCGACGGATGGAACCATCTCTATCTGTATGATACTACAGGTCGTCTGATCCGTCAGGTGACAAAAGG
GGAGTGGGAGGTTACAAACTTTGCAGGCTTCGATCCCAAGGGAACACGGCTCTATTTCGAAAGTACCGAAG
CCAGCCCTCTCGAACGCCATTTTTACTGTATTGATATCAAAGGAGGAAAGACAAAAGATCTGACTCCGGAG
TCGGGAATGCACCGCACTCAGCTATCTCCTGATGGTTCTGCCATAATCGATATTTTTCAGTCACCTACTGT
CCCGCGTAAGGTTACAGTGACAAATATCGGCAAAGGGTCTCACACACTCTTGGAGGCTAAGAACCCCGATA
CGGGCTATGCCATGCCGGAGATCAGAACGGGTACCATCATGGCGGCCGATGGGCAGACACCTCTTTATTAC
AAGCTCACGATGCCGCTTCATTTCGATCCGGCAAAGAAATATCCTGTTATTGTCTATGTTTACGGAGGACC
TCATGCCCAACTCGTAACCAAGACATGGCGCAGCTCTGTCGGTGGATGGGATATCTATATGGCACAGAAAG
GCTATGCCGTCTTTACGGTGGATAGTCGCGGATCTGCCAATAGAGGGGCTGCTTTCGAGCAGGTTATTCAT
CGTCGTTTGGGGCAGACCGAGATGGCCGATCAGATGTGCGGTGTGGATTTCCTCAAGAGCCAATCATGGGT
GGATGCCGATAGAATAGGAGTACATGGCTGGAGCTATGGTGGCTTTATGACTACGAATCTGATGCTTACGC
ACGGCGATGTCTTCAAAGTCGGAGTAGCCGGCGGGCCTGTCATAGACTGGAATCGATATGAGATTATGTAC
GGTGAGCGTTATTTCGATGCGCCACAGGAAAATCCCGAAGGATACGATGCTGCCAACCTGCTCAAACGAGC
CGGTGATCTGAAAGGACGACTTATGCTGATTCATGGAGCGATCGATCCGGTCGTGGTATGGCAGCATTCAC
TCCTTTTCCTTGATGCTTGCGTGAAGGCACGCACCTATCCTGACTATTACGTCTATCCGAGCCACGAACAT
AATGTGATGGGGCCGGACAGAGTACATTTGTATGAAACAATAACCCGTTATTTCACAGATCACTTATGA

Fig. 7d

INTERNATIONAL SEARCH REPORT

Inte· ›nal Application No
PCT/US 00/05551

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/57 C12N9/48 A61K39/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KIYAMA, M. ET AL.: "Sequence analysis of the Porphyromonas gingivalis dipeptidyl peptidase IV gene" BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1396, no. 1, 4 March 1998 (1998-03-04), pages 39-46, XP000925951 cited in the application	7
A	the whole document	1-6, 8-16, 18-20
	--- -/--	

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 October 2000	10.01.01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fuchs, U

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 00/05551

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Derwent Publications Ltd., London, GB; Class B04, AN 1990-053917 XP002149298 & JP 02 005880 A (SUNSTAR KK), 10 January 1990 (1990-01-10)	7
A	abstract	1-6, 8-16, 18-20

P,X	BANBULA, A. ET AL.: "Prolyl Tripetidyl Peptidase from Porphyromonas gingivalis" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 14, April 1999 (1999-04), pages 9246-9252, XP002149297 the whole document	1-16, 18-20

A	KABASHIMA, T. ET AL.: "Cloning, Sequencing, and Expression of the Dipeptidyl Peptidase IV Gene from Flavobacterium meningosepticum in Escherichia coil" ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol. 320, no. 1, 20 June 1995 (1995-06-20), pages 123-128, XP000925965 the whole document	1-16, 18-20

A	KURAMITSU, H.K.: "Proteases of Porphyromonas gingivalis: what don't they do?" ORAL MICROBIOLOGY AND IMMUNOLOGY, vol. 13, no. 5, October 1998 (1998-10), pages 263-270, XP000925947 abstract page 267, column 2, line 34 -page 268, column 1, line 58	1-16, 18-20

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/05551

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 15 and 16 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-16 AND 18-20 COMPLETELY

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-16 and 18-20 completely

An isolated prolyl tripeptidyl peptidase, active analog, active fragment, or active modification thereof having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids; an isolated prolyl tripeptidyl peptidase, active analog, active fragment, or active modification thereof being isolated from Porphyromonas gingivalis; an isolated polypeptide, active analog, active fragment, or active modification thereof having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids; an isolated polypeptide comprising an amino acid sequence having a percentage amino acid identity of greater than 35% with SEO ID NO: 30; an isolated nucleic acid fragment encoding a prolyl tripeptidyl peptidase, active analog, active fragment, or active modification thereof having amidolytic activity for cleavage of a peptide bond present in a target polypeptide having at least 4 amino acids, an isolated nucleic acid fragment encoding a polypeptide comprising an amino acid sequence having a percentage amino acid identity of greater than 35% with SEQ ID NO: 30; a method of identifying an inhibitor of a prolyl tripeptidyl peptidase, active analog, active fragment, or active modification thereof; a method of reducing growth of a bacterium comprising inhibiting a prolyl tripeptidyl peptidase, active analog, active fragment, or active modification thereof; an immunogenic composition comprising an isolated prolyl tripeptidyl peptidase, or an antigenic analog, antigenic fragment, or antigenic modification thereof, the prolyl tripeptidyl peptidase having amidolytic activity for cleavage of a peptide bond present in a target peptide having at least 4 amino acids; a composition comprising an inhibitor of an isolated prolyl tripeptidyl peptidase;

2. Claim : 17 partially and 21 completely

A method of reducing growth of a bacterium comprising inhibiting a prolyl dipeptidyl peptidase, active analog, active fragment, or active modification thereof; a dipeptidyl peptidase having an amino acid sequence comprising SEQ ID NO: 43;

3. Claim : 17 partially and 22 completely

A method of reducing growth of a bacterium comprising inhibiting a prolyl dipeptidyl peptidase, active analog, active fragment, or active modification thereof; a dipeptidyl peptidase having an amino acid sequence comprising SEQ ID NO: 44;

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

4. Claim : 17 partially and 23 completely

A method of reducing growth of a bacterium comprising inhibiting a prolyl dipeptidyl peptidase, active analog, active fragment, or active modification thereof; a dipeptidyl peptidase having an amino acid sequence comprising SEQ ID NO: 45.

INTERNATIONAL SEARCH REPORT

.nformation on patent family members

Inter nal Application No
PCT/US 00/05551

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 2005880 A	10-01-1990	NONE	